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Contents

Table of Figures

List of Abbreviations

AA	Arachidonic acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AMR	Ashwell-Morrell receptor
ANOVA	Analysis of variance
ASA	Aspirin
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
CRP-XL	Collagen related peptide - cross linked
CVD	Cardiovascular disease
DM	Diabetes mellitus
Gas6	Growth arrest-specific
GLUT1	Glucose transporter 1
GLUT3	Glucose transporter 3
GPVI	Glycoprotein VI
HT	HEPES Tyrode's
IGF-1	Insulin-like growth factor-1
IL-6	Interleukin 6
IP_3	Inositol-1,4,5-triphosphate
LDL	Low-density lipoprotein
MATE	Multidrug and toxin extruder
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MPTP	Mitochondrial permeability transition pore
NO	Nitric oxide

OAG	1-oleoyl-2-accetyl-sn-glycerol
OCT	Organic cation transporters
PAR	Protease activated receptor
PGE1	Prostaglandin E1
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B (also known as AKT)
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
PRP	Platelet rich plasma
PS	Phosphatidylserine
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
SEM	Standard error of the mean
SLC	Solute carrier
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TMRM	Tetramethylrhodamine
TPO	Thrombopoietin
TxA_2	Thromboxane A_2
UA	Unstable angina
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
$\Delta\Psi m$	Change in mitochondrial membrane potential

Abstract

Background and Objectives: Platelets play a vital role in haemostasis but if activated inappropriately can contribute to cardiovascular disease (CVD) and result in myocardial infarction (MI) and/or stroke. Platelet hyperreactivity is linked to several diseases that are risk factors for CVD, including diabetes. The underlying cause of platelet hyperreactivity in diabetes is still largely unknown. The aim of this study was therefore (i) to investigate the effects of hyperglycaemia and thrombopoietin (TPO) on platelet function and (ii) to determine whether the anti-diabetes treatments, metformin and rosiglitazone affect platelet function. *Experimental design:* The effect of 100 ng/mL TPO, 25 mM glucose, 40 μM metformin and 100 μM rosiglitazone on platelet function were investigated. The functional assays include CRP-XL, PAR1-AP or thrombin stimulated platelet aggregation, integrin activation, phosphatidylserine exposure, reactive oxygen species generation, mitochondrial membrane potential depolarisation and calcium signalling. *Results:* Glucose alone had no effect on platelet aggregation but enhanced mitochondrial membrane depolarisation and PS exposure. TPO significantly enhanced platelet aggregation, mitochondrial membrane depolarisation, ROS generation and PS exposure. Metformin had no significant effect on platelet aggregation, whereas Rosiglitazone significantly reduced platelet aggregation but also significantly increased PS exposure and ROS generation. *Conclusion:* Hyperglycaemia and TPO contribute to a pro-coagulant platelet phenotype via increased PS exposure, providing a potential explanation for the platelet hyperreactivity observed in patients with diabetes and other diseases associated with chronic hyperglycaemia and elevated serum levels of TPO. Rosiglitazone has both anti-platelet and pro-coagulant properties. The latter of which, with further research, could provide an explanation behind the increase risk of CVD observed in rosiglitazone treated patients.

Introduction

General Introduction

Cardiovascular diseases (CVD) are disorders of the heart and blood vessels and are the most common cause of morbidity and mortality in the world, causing approximately 17.9 million deaths worldwide every year (WHO, 2017). Some examples of CVD are; cerebrovascular disease, coronary heart disease and peripheral arterial disease. There are several risk factors that enhance the likelihood of developing CVD and a vast majority of them are lifestyle choices e.g. smoking, obesity, fatty diet, high cholesterol, high blood pressure and physical inactivity. However, other non-lifestyle caused factors include genetic predisposition factors and diabetes (World Heart Federation, 2017).

Myocardial infarction (MI) and stroke are the main cause of death for patients with CVD and they are usually as a direct effect of atherosclerosis, which is when the build-up of atheroma causes narrowing of the arteries and prevention of blood, and therefore oxygen, reaching the heart or brain. Platelets play an important role in haemostasis and contribute to events which can lead to thrombosis and atherosclerosis. Therefore, in patients with an already increased risk of developing CVD, platelet hyperreactivity is potentially life threatening.

Platelet activation

Platelets are the smallest cellular component of the blood and are produced by megakaryocytes in the bone marrow. Platelets initiate the haemostatic process and play a major role in the development of arterial thrombosis. At the site of vascular injury platelets bind to sub endothelial components such as collagen, which are otherwise hidden, which in turn induces platelet activation and fibrin formation (Fig. 1). This is an important process that is required to prevent excessive blood loss. However, platelet activation and thrombus formation can be a life-threatening process if it occurs inappropriately. For example, at the site of atherosclerotic plaque rupture where platelet aggregation leads to clot formation which can in turn lead to vascular occlusion and ischemic infarction.

Under normal circumstances, platelets circulate the body through vessels with an intact, healthy endothelium in their original, inactivated state. Endothelium production of nitric oxide (NO) and prostacyclin and the absence of platelet activating factors supports this state. However, at the site of endothelial injury, the endothelium is no longer inhibiting platelet function and platelets are exposed to sub endothelial components such as collagen which binds to platelets (Fig. 1). Platelet activation occurs immediately after platelet adhesion and results in numerous functional changes including; exocytosis of secretory granules, shape change and activation of integrins. The $\alpha_{IIb}\beta_3$ integrin is expressed on the

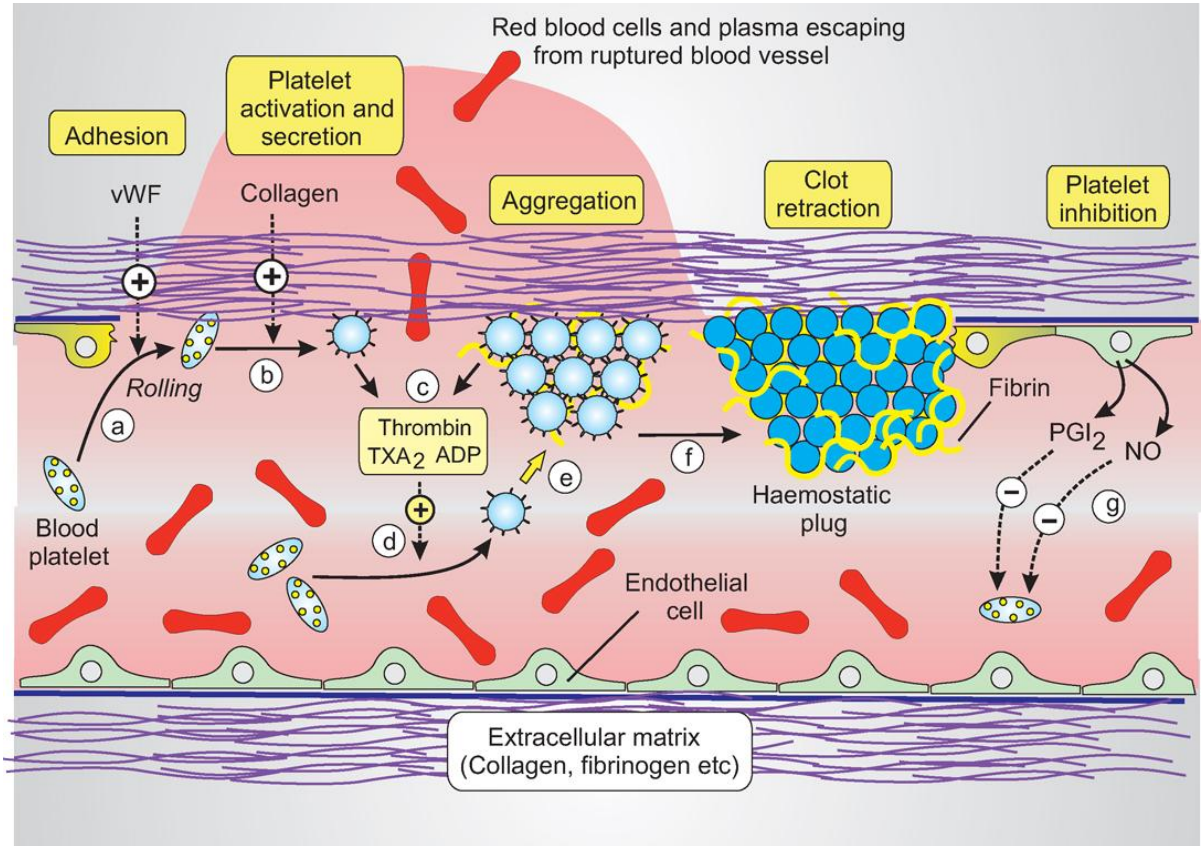

Figure 1: Thrombus formation. At the site of vascular injury platelets adhere to subendothelial components such as collagen and Von Willebrand factor (a) resulting in platelet activation and secretion (b) of platelet mediators such as thrombin, thromboxane A2 and ADP (c) which results in additional activation (d) and recruitment of platelets (e) fibrils of fibrin form around the aggregation providing stability (f) and formation of the haemostatic plug. Intact endothelium will no longer be exposing platelet activating molecules and will also be releasing, prostacyclin and NO (g) which will inhibit further platelet activation. Figure from (Berridge, 2014; Natarajan, Zaman & Marshall, 2008).

surface of platelets and when a platelet is activated this integrin is converted to a conformational state that has high affinity for fibrinogen from one with a low affinity for fibrinogen (Fig. 2). Fibrinogen can bind to two receptors at a time and is therefore able to cross-link platelets, ultimately resulting in platelet aggregation. These activated integrins are also able to bind to sub endothelial von Willebrand factor (vWF) to further anchor the platelets and provide additional clot stabilisation (Ginsberg, 2014).

There are several different receptors on the surface of the platelet that when bound to by an agonist will initiate platelet activation via a wide variety of signalling pathways. Platelets can respond to an array of mediators since they express such a large, diverse and dynamic range of receptors from at least 9 protein super-families (Fig. 2). For this study I will mainly be focusing on protease activated receptor 1 activating peptide (PAR1-AP), collagen-related peptide-cross-linked (CRP-XL) and thrombin-stimulated platelet activation.

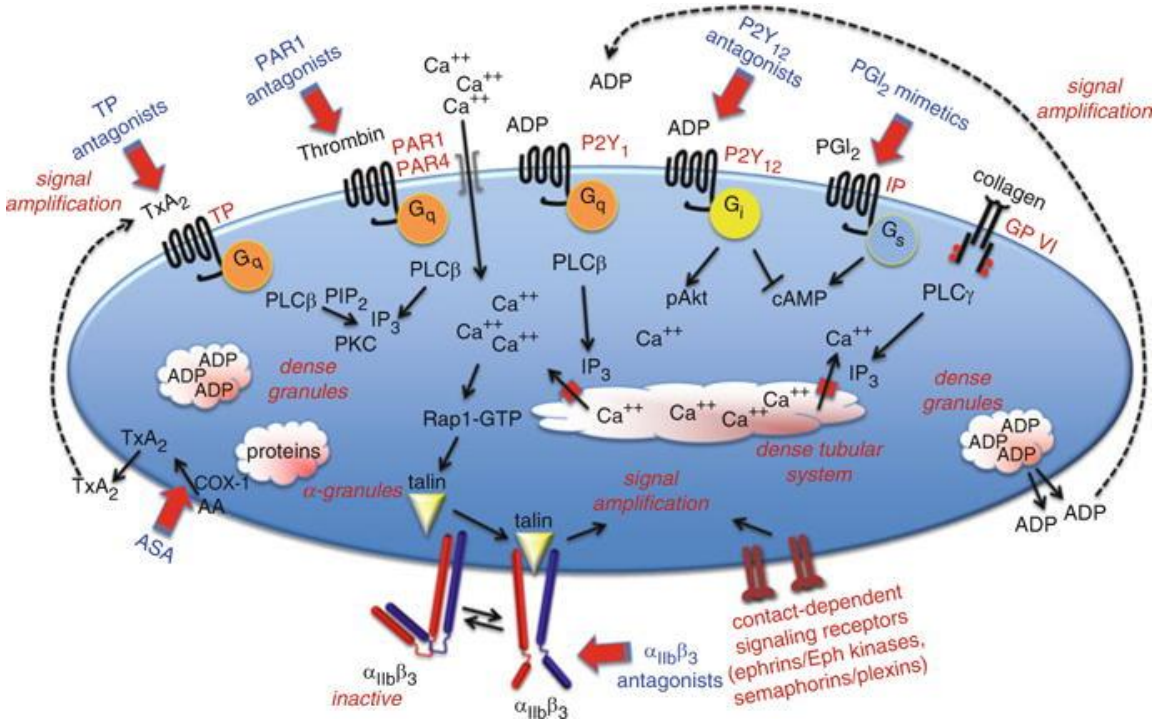

Figure 2: An outline of the main receptors and signalling pathways involved in platelet activation including the receptors for CRP-XL (GPVI), PAR1-AP (PAR1) and thrombin (PAR1/PAR4). PLC, phospholipase C. PKC, protein kinase C. IP_3, inositol-1,4,5-trisphosphate. TxA_2, thromboxane A_2. AKT, protein kinase B. cAMP, cyclic adenosine monophosphate. COX-1, cyclooxygenase-1. ADP, adenosine diphosphate. AA, arachidonic acid. ASA, aspirin. Figure from: (Stalker, Newman, Ma, Wannemacher & Brass, 2012)

Phosphatidylserine exposure

Phosphatidylserine (PS) is a phospholipid and a component of the cell membrane. In the plasma membrane of a cell, phospholipids are not homogeneously spread across both leaflets of the membrane bilayer. In a resting cell (non-activate, non-apoptotic) neutral, choline-containing phospholipids such as: phosphatidylcholine and sphingomyelin are located on the outer leaflet. Whereas the negatively-charged phospholipids including: phosphatidylinositol and PS are actively held on the inner (cytosolic side) leaflet of the cell membrane (Bevers, Comfurius & Zwaal, 1983; Bevers & Williamson, 2010). However, under certain conditions such as when a cell undergoes apoptosis these negatively charged phospholipids are no longer restricted to the inner leaflet resulting in a large proportion of the PS being exposed on the outer leaflet this is known as the scrambling process (van Kruchten et al., 2013).

PS exposure in platelets has predominately been associated with apoptosis (Gyulkhandanyan, Mutlu, Freedman & Leytin, 2012). It has also been reported that platelets stimulated with both CRP-XL and thrombin can lead to a platelet phenotype that is associated with increased PS exposure and enhanced platelet coagulation (Lentz, 2003).

Dual stimulated platelet activation leads to significantly increased cytosolic calcium concentration which in turn results in mitochondrial overload and the formation of mitochondrial permeability transition pores (MPTP). An increase in MPTP formation causes a reduction in mitochondrial membrane integrity and an increase in mitochondrial membrane depolarisation which results in a decrease in ATP and an increase in reactive oxygen species (ROS) generation. Mitochondrial membrane depolarisation can also lead to even further increases in cytosolic calcium levels, PS exposure and a reduction in plasma membrane integrity (Jackson & Schoenwaelder, 2010). There are two pathways which regulate PS exposure. Agonist stimulation leads to a calcium-dependent, caspase-independent pathway resulting in PS exposure and this form of PS exposure is essential for platelet thrombin generation. Whereas the Bak/Bax-caspase-mediated PS exposure pathway is independent of platelet activation and is associated with necrosis (Schoenwaelder et al., 2009). Scott syndrome is a rare bleeding disorder, characterised by delayed haemostasis and wound healing, this is caused by the absence of the transmembrane protein 16F which leads to disrupted scramblase activity and the inability to expose PS (Zwaal, Comfurius & Bevers, 2004). At present, the only treatment available for patients with Scott syndrome is transfusion of normal platelets. Scott syndrome highlights the importance of PS exposure in haemostasis.

Platelet exposure of PS is an important part of the blood coagulation process. Blood coagulation occurs at the same time as platelet activation and includes a series of inactive factors being converted to an active proteolytic enzyme which results in the conversion of prothrombin to thrombin. Thrombin is then responsible for converting fibrinogen to fibrin. As the fibrils of fibrin form, activated platelets are trapped, forming a clot (Wolberg, 2007). Coagulation reactions also enhance platelet activation by releasing thrombin. Platelet activation and coagulation systems tend to be mutually reinforcing with each inducing the other to form the final clot. Although agonist-induced PS exposure exhibits the same characteristics as apoptotic platelets, the mechanism that regulates their formation is different. At present it is unclear whether PS exposure in apoptotic platelets has any relevance to thrombin generation in vivo. It is likely that the externalisation of PS in apoptotic platelets acts as a recognition determinant for clearance by phagocytosis, as it does in other cells such as lymphocytes (Fadok, Voelker, Campbell, Cohen, Bratton & Henson, 1992).

Platelet hyperreactivity

Overly reactive platelets can be linked to several different diseases and there is increasing evidence to suggest that increased platelet reactivity can identify patients that are at greater risk of CVD. There are numerous studies that have linked increased platelet

function with worse clinical outcomes in various cardiovascular settings (Kabbani, Watkins, Ashikaga, Terrien, Sobel & Schneider, 2003; Puurunen et al., 2018; Trip, Cats, van Capelle & Vreeken, 1990). Platelet hyperreactivity has been associated with several diseases including; unstable angina (UA), chronic kidney disease, chronic obstructive pulmonary disease, peripheral arterial disease and diabetes (Gremmel et al., 2013; Maclay et al., 2011; Robless, Okonko, Lintott, Mansfield, Mikhailidis & Stansby, 2003); (Gaiz, Mosawy, Colson & Singh, 2017; Lupia et al., 2006)

Diabetes Mellitus

Overview

Diabetes mellitus (DM) is a chronic disease and it is estimated that 1 in 11 adults worldwide are diagnosed with DM (Zheng, Ley & Hu, 2018). DM occurs when the pancreas doesn't produce enough insulin (type 1) or the body cannot respond effectively to insulin (type 2). Insulin is a hormone that is released from the pancreas and is important for controlling blood sugar levels. Under normal circumstances an increase in blood glucose directly results in the release of insulin from β cells within the islets of Langerhans, located in the pancreas. Insulin is the most potent anabolic hormone and once released into the blood stream it promotes the synthesis and storage of carbohydrates, lipids and proteins whilst also inhibiting their degradation and release into the circulation. Insulin stimulates the uptake of glucose into cells and enhances the expression and activity of glycogen synthesis enzymes. Ultimately leading to a reduction in the concentration of glucose in the bloodstream.

Type 1 DM (T1DM) is a disease that is caused by the autoimmune destruction of the insulin-producing β-cells. In T1DM patients, when blood glucose levels are increased, insulin is not released and the patient is subjected to chronic hyperglycaemia, unless otherwise treated (Atkinson, 2012). T1DM is characterized by the inflammation of the islets of Langerhans and β-cell damage, it is currently unclear why the autoimmune destruction is specific to the insulin secreting β-cells (Atkinson et al., 2011).On the other hand, Type 2 DM (T2DM) is due to impaired insulin release as well as insulin resistance (Kahn, Cooper & Del Prato, 2014). T2DM is caused by the combination of genetic and environmental factors which result in impaired insulin secretion and insulin resistance. Environmental factors that are known to contribute to T2DM include: obesity, lack of exercise, stress and aging. T2DM begins with insulin resistance which results in an increase in insulin secretion and glucose levels are maintained, this only then becomes a problem when the β-cells are no longer able to increase insulin secretion to overcome the ever-increasing insulin resistance. Therefore patients with T2DM are likely to have higher absolute insulin levels compared to a

healthy control, although this is still lower than necessary due to the degree of insulin resistance (Skyler et al., 2017).

It is a well-established fact that DM is a major risk factor for CVD (World heart federation, 2017), and risk factors associated with T2DM are very similar to CVD risk factors. Approximately 65% of all diabetic deaths are due to CVD and the risk of cardiovascular mortality is 2-4 times higher in patients with DM in comparison to non-diabetics (Deshpande, Harris-Hayes & Schootman, 2008). CVD is the most common cause of morbidity and mortality in diabetic patients and accelerated atherosclerosis is thought the be the main underlying factor that contributes to the high risk of CVD incidence (Nathan, 1993). Diabetic angiopathy involves inflammation, atherosclerosis and thrombus formation all of which platelets play a vital role in. The pathogenesis and increased risk of CVD in diabetes cannot simply be explained by the co-existence of the classic risk factors such as smoking, hypertension and obesity but can be explained by an increase in platelet reactivity seen in patients with DM. Table 1 lists the main platelet abnormalities seen in diabetic patients (Natarajan, Zaman & Marshall, 2008; Papazafiropoulou, Papanas, Pappas, Maltezos & Mikhailidis, 2015). In summary, platelets in patients with DM are hyperreactive.

Table 1: Platelet abnormalities in diabetes
Reduced membrane fluidity, altered platelet shape, secretion and aggregation
Increased granule secretion
Disordered calcium homeostasis
Increased thromboxane A2 production from arachidonic acid
Decreased prostacyclin production
Increased expression of platelet surface adhesion molecules and receptors
Increased platelet-dependent activation of coagulation

Platelet hyperreactivity is arguably an important factor promoting increased risk of CVD in patients with diabetes - with an increase in the number of activated circulating platelets leading to an enhanced likelihood of thrombus formation, occlusion, and life-threatening complications. It is widely accepted that diabetic platelets are hyperreactive (Vinik, Erbas, Park, Nolan & Pittenger, 2001), however the underlying cause/mechanism is less well understood. There are several theories as to what may cause this increase in platelet activity in patients with diabetes including:

- Oxidative stress and inflammation
- Insulin resistance and insulin deficiency
- Metabolic abnormalities

Oxidative stress and inflammation are both associated with DM and it has been demonstrated that they contribute to increased platelet reactivity (Calverley et al., 2003; Salvemini, de Nucci, Sneddon & Vane, 1989). Oxidative stress has been shown to reduce NO and prostacyclin production by impairing endothelial function (Schäfer & Bauersachs, 2008).

A few studies have suggested that loss of platelet insulin sensitivity is a cause of platelet hyperreactivity and demonstrate that platelet activation is antagonised by insulin (Westerbacka et al., 2002). Therefore, implying that in patients who have decreased insulin levels and increased insulin resistance, their platelets are no longer subjected to insulin-mediated antagonism and platelet reactivity is increased. However, as previously discussed, patients with T2DM actually have increased actual levels of insulin in circulation and therefore if insulin does inhibit platelet activation this would suggest that in patients with T2DM platelet activity would be reduced - which is not the case. Additionally, a number of other studies have demonstrated that insulin does not reduce platelet function. Growth factors related to insulin actually increase platelet function and deletion of the insulin receptor in mouse platelets resulted in reduced aggregation, granule secretion and integrin $\alpha_{IIb}\beta_3$ activation (Moore et al., 2015). It has also been confirmed that there is relatively low insulin receptor expression on human platelets and that insulin, therefore, has a minimal effect on platelet function (Hunter & Hers, 2009). Therefore, concluding that a reduction in serum levels of insulin does not cause platelet hyperreactivity observed in diabetes.

There are several metabolic conditions that are associated with DM including; obesity, dyslipidaemia and hyperglycaemia. The present study focuses on investigating the effects of hyperglycaemia on platelet hyperreactivity.

Hyperglycaemia

A blood glucose concentration of 5.5 mmol/L or lower is considered normal during fasting and a concentration of less than 7.8 mmol/L 2 hours post prandial is considered normal. Whereas a typical diabetic blood glucose concentration during fasting is > 7 mmol/l and > 11.1 mmol/l 2 hours post prandial (The Global Diabetes Community, 2018). Hyperglycaemia is the main diabetic phenotype and diabetic platelets will be exposed to chronic hyperglycaemia. There are many ways in which this could be the cause of platelet hyperreactivity in DM patients with short term glycaemic control has been shown to reduce spontaneous platelet aggregation in patients with T2DM (Hara, Omori, Sumioka & Aso, 2012).

There have been several studies conducted with the aim of investigating the effect of hyperglycaemia on platelet function, table 2 summarises a few of their findings. In general, studies into the effect of hyperglycaemia on platelet function seem to produce a varied range of results. For example, some studies show that hyperglycaemia can enhance platelet aggregation, but others show no effect of hyperglycaemia on platelet aggregation. Several studies that have shown high glucose treatment enhances platelet aggregation repeated their experiments in the presence of the iso-osmolar control, mannitol, instead of high glucose and found this showed the same effect. Therefore, any effect of hyperglycaemia could purely be due to an increase in osmolarity (Keating, Sobel & Schneider, 2003; Sudic, Razmara, Forslund, Ji, Hjemdahl & Li, 2006). On the other hand, studies using the same control have concluded that the effects of glucose are not due to an increase in osmolarity (Russo et al., 2012). Conversely, there is little variation in the literature regarding the ability of glucose to rescue the inhibitory effects of aspirin. The majority of studies dedicated to investigating this have shown that the presence of a high concentration of glucose results in a decrease in the inhibition of platelet aggregation induced by aspirin (Kobzar, Mardla & Samel, 2011; Le Guyader, Pacheco, Seaver, Davis-Gorman, Copeland & McDonagh, 2009; Russo et al., 2012).

Platelets express glucose transporter 1 (GLUT1) and glucose transporter 3 (GLUT3) (Craik, Stewart & Cheeseman, 1995) which mediate glucose entry into the platelet via facilitative diffusion. GLUT3 is the more abundant glucose transporter in platelets and approximately 85% is located on α-granule membranes and 15% is located on the platelet plasma membrane (Heijnen, Oorschot, Sixma, Slot & James, 1997). Uptake of glucose into platelets is enhanced when platelets are activated (Karpatkin, 1967). Platelet activation and secretion leads to the translocation of α granules to the plasma membrane which results in increased expression of GLUT3 at the platelet plasma membrane thereby facilitating further glucose uptake (Fidler et al., 2017).

Kraakman et al proposed a slightly alternative theory which could explain the hyperreactivity of platelets in patients with DM (Kraakman et al., 2017). In this study Kraakman et al suggested that hyperglycaemia causes neutrophils in the bloodstream to release S100 calcium-binding protein A8/A9 which would in turn bind to the receptor for advanced glycation end products (RAGE) on Kupffer cells. This results in the release of interleukin 6 (IL-6) which causes TPO secretion from hepatocytes. Increased TPO leads to increased platelet production which ultimately results in greater numbers of reticulated platelets. They showed that reticulated platelets are more reactive than mature platelets, which could explain platelet hyperreactivity in diabetic patients. This is an interesting theory but it does not explain any direct effect of glucose on platelet function.

Table 2: Brief outline of some studies that have investigated the effects of glucose on platelet function		
Title	**Hyperglycaemic effect reported**	**Reference**
High glucose levels enhance platelet activation: involvement of multiple mechanisms	- Glucose increased thrombin stimulated platelet P-selectin and fibrinogen binding - L-glucose, sucrose and galactose produced similar enhancements as D-glucose, suggesting that elevated osmolarity is what triggers the changes in platelet signalling	(Sudic, Razmara, Forslund, Ji, Hjemdahl & Li, 2006)
Glucose and collagen regulate human platelet activity through aldose reductase induction of thromboxane	- Collagen stimulated aggregation was enhanced when the platelets were pre-incubated with high glucose - Concentration-dependent effect of glucose on collagen stimulated P selectin exposure	(Tang et al., 2011)
Glucose impairs aspirin inhibition in platelets through a NAD(P)H oxidase signalling pathway	- Glucose has no effect on ADP induced platelet aggregation - Glucose reversed aspirin inhibition of ADP-stimulated aggregation and ROS generation in a dose-dependent way	(Kobzar, Mardla & Samel, 2017)
Effects of increased concentrations of glucose on platelet reactivity in healthy subjects and in patients with and without diabetes	- Exposure of both healthy and diabetic platelets to an increase in glucose resulted in an increase in P selectin exposure and integrin activation - - Same effect was seen in the presence of the metabolically inactive mannitol so they concluded that the increase in platelet reactivity was due to an increase in osmolarity	(Keating, Sobel & Schneider, 2003)
Hyperglycaemia-induced platelet activation in type 2 diabetes is resistant to aspirin but not to a nitric oxide donating agent	- Arachidonic acid induced platelet aggregation was unaffected by acute hyperglycaemia - P selectin expression on platelets was unaffected by acute hyperglycaemia - Acute hyperglycaemia enhanced shear stress induced platelet activation and this was resistant to aspirin inhibition	(Gresele et al., 2010)
Short term exposure of platelets to glucose impairs inhibition of platelet aggregation by cyclooxygenase inhibitors	- 22.4 mM glucose impaired aspirin inhibition of ADP, arachidonic acid, collagen and thrombin stimulated platelet aggregation - Glucose alone had no effect on platelet aggregation	(Kobzar, Mardla & Samel, 2011)
High glucose inhibits the aspirin-induces activation of the nitric oxide /cGMP /cGMP-dependent protein kinase pathway and doesn't affect the aspirin-induced inhibition of thromboxane synthesis in human platelets	- Platelet exposure to 25 mmol/L glucose reduced the ability of aspirin to inhibit platelet aggregation - Glucose had no effect on the ability of aspirin to inhibit thromboxane synthesis - Hyperglycaemia prevented the aspirin-induced activation of the NO/cGMP/PKG pathway - No effect of the iso-osmolar mannitol was seen	(Russo et al., 2012)

Platelet primers

It is well documented that increased levels of circulating primers can induce platelet hyperreactivity (Gresele, Falcinelli & Momi, 2008; Lupia et al., 2009; Stolla, Li, Lu & Woulfe, 2013). A platelet primer cannot stimulate platelet aggregation by itself, but can enhance platelet functional responses induced by agonist (e.g. PAR1-AP, CRP-XL etc) stimulation. Table 3 lists the main known platelet primers (Gresele, Falcinelli & Momi, 2008). This study will predominantly focus on the ability of TPO to induce platelet hyperreactivity, as TPO serum levels have been shown to be significantly higher in patients with DM.

Table 3: Platelet primers

Proteins:	
Gas6	MMP-1 / MMP-2
Clotting FVIII	CD40L
Bile salt-dependent lipase	LDL
Cytokines and Chemokines:	
VEGF	Macrophage-derived chemokine
Thrombopoietin	Stromal cell-derived factor 1
Thymus activation-regulated chemokine	Leptin
Hormones:	
17β-estradiol	Histamine
Epinephrine	IGF-1
Prostaglandins:	
PGE_2	

Thrombopoietin

TPO is a humoral growth factor that is produced by the liver and kidneys. It is removed from circulation when it binds to its receptor, c-Mpl, which is present on both megakaryocytes and platelets (Li, Xia & Kuter, 1999). Upon binding to the c-Mpl receptor on megakaryocytes, TPO induces proliferation and production of platelets (Fig. 3) (Kaushansky, 1995) . TPO is therefore of particular pharmacological interest for the treatment of thrombocytopenia (Kuter, 2009). In this role, TPO is well defined, however less is known about the ability of TPO to act directly on platelets. TPO is a known platelet primer,

which has been demonstrated to enhance platelet functional responses induced by platelet agonists (van Willigen, Gorter & Akkerman, 2000).

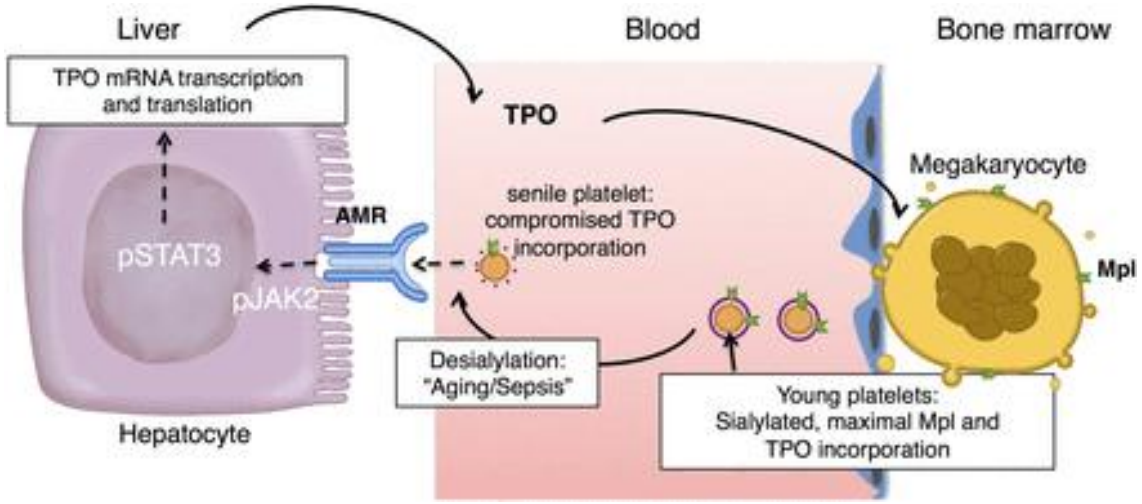

Figure 3: Thrombopoietin dependent platelet production. Megakaryocytes produce platelets, which when initially released into the bloodstream maximally internalise TPO. As the platelets become more mature they become less sensitive to TPO. Mature platelets are recognised by the hepatic Ashwell-Morrell receptor (AMR) and are ingested by hepatocytes. The ingestion of mature platelets results in hepatic release of TPO into the bloodstream. TPO binds to the c-Mpl receptor on megakaryocytes and stimulates the production of platelets. (Grozovsky, Giannini, Falet & Hoffmeister, 2015; Natarajan, Zaman & Marshall, 2008).

It has also been shown that TPO can enhance firm platelet adhesion to vWF, indicating that TPO might play an important role in thrombus formation (Van Os et al., 2003). TPO has been shown to enhance platelet functional responses via a phosphoinositide 3-kinase (PI3K)-dependent mechanism (Pasquet et al., 2000).

Serum levels of platelet primers such as tissue factor, insulin growth factor-1 (IGF-1) and TPO have been shown to be enhanced in patients with DM (Gerrits, Koekman, van Haeften & Akkerman, 2010; Grove, Hvas, Mortensen, Larsen & Kristensen, 2011; Stolla, Li, Lu & Woulfe, 2013). Grove et al (Grove, Hvas, Mortensen, Larsen & Kristensen, 2011) demonstrated that TPO levels in the blood are significantly higher in patients with diabetes compared to healthy controls. Lupia et al (Lupia et al., 2006) investigated the potential role of elevated levels of TPO in platelet activation during UA. This study showed that patients with UA had significantly higher TPO serum levels in comparison to healthy controls and UA patients platelets expressed elevated numbers of the TPO receptor c-Mpl. Lupia et al (Lupia et al., 2006) suggest that TPO may increase platelet activation in patients suffering from acute angina and ultimately contribute to the pathogenesis of acute coronary syndrome. An aim of this study was to investigate whether TPO plays a role in promoting platelet hyperreactivity in diabetic platelets and whether platelet reactivity could be further enhanced when exposed to elevated TPO in combination with hyperglycaemia.

Anti-diabetic treatment

Overview

The symptoms associated with DM put patients at high risk of long-term macro- and micro-vascular complications, which if not treated, could lead to death. There are several different treatment options available depending on the type of diabetes and the individual. For patients diagnosed with T1DM treatment is focused on increasing insulin levels usually via a self-administered insulin injection. In patients diagnosed with T2DM, because they have a reduced response to insulin, the therapeutic focus is on preventing complications caused by hyperglycaemia. The main classes of oral anti-diabetic medication used to treat T2DM include; sulfonylureas, biguanides, thiazolidinediones, meglitinide, dipeptidyl peptidase 4 (DPP-A) inhibitors, sodium-glucose cotransporter inhibitors and α-glucosidase inhibitors (Chaudhury et al., 2017).

As well as the 'normal' symptoms of diabetes the increased risk of CVD also needs to be treated. CVD risk can be greatly reduced with anti-platelet therapy in non-diabetic patients. However, anti-platelet therapies are generally less efficacious in diabetic patients (Angiolillo, 2009). Patients who are resistant to antiplatelet therapies have been shown to have hyperreactive platelets (Angiolillo et al., 2005; Dichiara et al., 2007; Macchi et al., 2002). It is possible that the resistance to antiplatelet treatment shown by diabetics is due to the hyperreactive platelet phenotype. Therefore, it is imperative that an alternative antiplatelet therapy for patients with DM is investigated.

Coincidently, a few anti-diabetic treatments, the oral hypoglycaemic agents such as the biguanides, sulfonylureas and glitazones in particular, also show antiplatelet properties (Ishizuka et al., 1998; Papazafiropoulou, Papanas, Pappas, Maltezos & Mikhailidis, 2015; Xiao et al., 2015). Numerous studies have investigated the effects of diabetes treatment on platelet function, but the results have been variable. This study will focus on the effects of metformin and rosiglitazone on platelet function.

Metformin

Metformin is an oral anti-diabetic that helps to control blood sugar levels and is the first line medication for the treatment of T2DM. Metformin is an example of a biguanide and it works by activating adenosine monophosphate-activated protein kinase in the liver, which in turn causes hepatic uptake of glucose and inhibition of gluconeogenesis.

It has been reported that metformin reduces mortality and diabetes-associated thrombotic complications (Lu et al., 2014; Roussel et al., 2010). There are a few studies investigating the effects of metformin therapy on platelet function. Xin et al (Xin et al., 2016) showed that metformin inhibits platelet activation both in vivo and in vitro. They suggest that

metformin does this by lowering extracellular mitochondrial DNA release which can supposedly act as an agonist to induce platelet activation and thrombosis. Metformin has also been shown to reduce oxidative stress and limit platelet activation in a study with newly diagnosed T2DM patients (Formoso et al., 2008). However, it has been suggested that any effect metformin has on platelet function is an indirect effect, based on the ability of metformin to reduce glucose levels rather than any direct effect on platelets (Collier, Watson, Patrick, Ludlam & Clarke, 1989).

Metformin is a cationic drug and therefore depends on transporters such as; organic cation transporters (OCTs) and multidrug and toxin extruders (MATEs) for its pharmacokinetic movement across the cell membrane (Pakkir Maideen, Jumale & Balasubramaniam, 2017). OCT proteins are members of the solute carrier family, subfamily 22 (SLC22). According to the human proteome map both SLC22A1 (OCT1) and SLC22A18 are present in platelets (Fig. 4). It is therefore reasonable to suggest that metformin may well be capable of getting across the platelet cell membrane and having a direct effect on platelet function. Therefore, further investigation into the mechanism of action of metformin and its ability to affect platelet function is required.

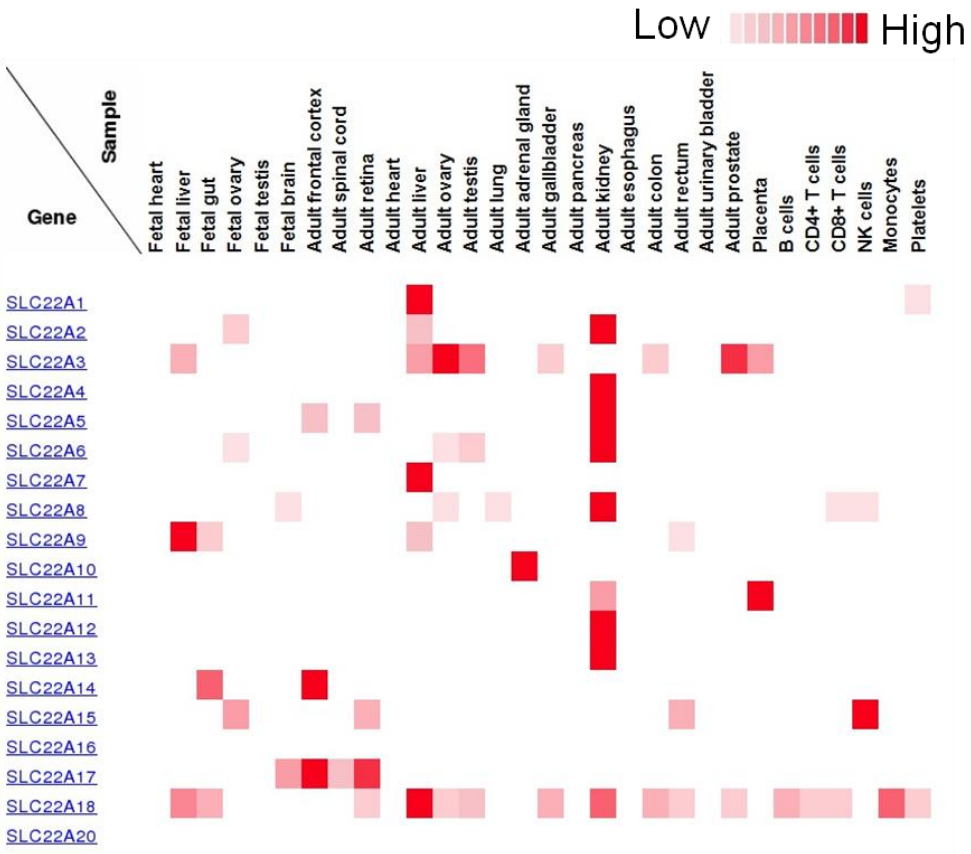

Figure 4: Human proteome map of solute carrier family, subfamily 22. The human proteome map, analysed by total proteome mass spectrometry (reference), was queried for the SLC22A protein family and the relative abundance of each protein is shown in a heat map. White represents little or no protein and dark red represents high abundance. SLC22A1 = OCT1. Figure adapted from: http://www.humanproteomemap.org/batch.php.

Rosiglitazone

Rosiglitazone is an example of a thiazolidinedione and acts as an insulin sensitizer by improving insulin action. Following a meta-analysis published in 2007 that linked rosiglitazone use to an increased risk of heart attack rosiglitazone has been withdrawn from the European market but it remains available in the US (Nissen & Wolski, 2007). Rosiglitazone is an agonist at the peroxisome proliferator-activated receptors (PPAR), a group of receptors that belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors, with the greatest specificity for PPARγ. Upon activation, PPARγ facilitates increased glucose uptake in numerous tissues including adipose, muscle and liver. Platelets are known to express PPARγ (Akbiyik, Ray, Gettings, Blumberg, Francis & Phipps, 2004) and both natural and synthetic PPARγ ligands have been shown to inhibit platelet function, thrombosis and haemostasis (Unsworth, Flora & Gibbins, 2018). Sidhu et al showed that rosiglitazone reduces platelet activity in non-diabetic patients with coronary artery disease (Sidhu, Cowan & Kaski, 2004) and Khanolkar et al showed that rosiglitazone further reduced platelet aggregation and oxidative stress in patients with T2 DM (Khanolkar et al., 2008).

A recent study has shown that PPAR ligands, such as rosiglitazone, modulate the activity of the GPVI collagen receptor-stimulated signalling pathway. Which in turn results in inhibited platelet activation, aggregation and thrombus formation (Moraes et al., 2010). It has also been suggested that the anti-platelet effects of rosiglitazone are AMP-activated protein kinase (AMPK) mediated (Liu, Park, Chang, Huh, Lee & Lee, 2016). AMPK is an enzyme that is known to play a role in cellular energy homeostasis. Liu et al found that rosiglitazone stimulated both AMPK and PPARγ in platelets. And although stimulation of either lead to reduced platelet aggregation the efficacy attributable to AMPK activation was much greater than that caused by PPARγ stimulation (Liu, Park, Chang, Huh, Lee & Lee, 2016).

Aims

Platelet hyperreactivity in diabetes is likely to lead to the acceleration of a cardiac event and it is important that the mechanisms underlying such hyperreactivity are investigated. This project has four main aims. Firstly, to investigate the effects of hyperglycaemia on platelet function, including aggregation, PS exposure, mitochondrial membrane potential, ROS generation and calcium signalling. Secondly, to extend previous studies by showing the effects of the platelet primer, TPO, on aggregation, PS exposure, ROS generation and mitochondrial membrane potential. Thirdly, to determine whether hyperglycaemia affects the ability of TPO to enhance platelet function. And finally, the fourth aim was to study whether anti-diabetic treatments are able to reverse the hyperreactive qualities induced by TPO and/or hyperglycaemia.

Materials and Methods

Materials

Agonists, Antagonists and Inhibitors

Protease-activated receptor 1 (PAR-1)-activating peptide (SFLLRN-NH_2) was from Bachem (Switzerland). Crosslinked collagen-related peptide (CRP-XL) was from Richard Farndale (University of Cambridge). Human recombinant TPO, aspirin, ARC66096 tetrasodium salt, rosiglitazone and metformin were from Bio-techne (Abingdon, UK). D-glucose was from ThermoFisher Scientific (Loughborough, UK). Insulin-like growth factor-1 (IGF-1) was from Immunological and Biochemical Test Systems (Binzwangen, Germany). Apyrase, prostaglandin E1 (PGE1), indomethacin, mannitol and thrombin were all sourced from Sigma Aldrich (Poole, UK).

Flow cytometry and calcium assay antibodies and dyes

FITC-conjugated PAC-1 and PE-conjugated CD62P antibodies were from BD Biosciences (Berkshire, UK). Annexin V-Alexa Fluor 488 conjugate, Tetramethylrhodamine (TMRM), CM-H2DCFDA (general oxidative stress indicator) and Fura-2 AM cell permanent calcium indicator were from Thermo Fisher Scientific (Loughborough, UK).

Buffers and Solutions

Buffer/Solution	**Composition**
ACD	85 mM trisodium citrate dihydrate, 71 mM citric acid, 111 mM D-glucose
HEPES-Tyrode (pH 7.2)	10 mM HEPES, 145 mM sodium chloride, 3 mM potassium chloride, 0.5 mM sodium dihydrogen phosphate, 1 mM magnesium sulphate 7-hydrate
Anticoagulant	4 % trisodium citrate dihydrate dissolved in purified H2O
EGTA	100 mM EGTA, 10 mM HEPES
Tris, pH 7.4	500 mM Tris Base
Triton	20 % triton dissolved in H2O
Trisodium citrate	Dissolved in Mili-Q water to 4% w/v.

Unless otherwise stated, all other reagents were from Sigma Aldrich (Poole, UK).

Platelet Preparation:

Venous blood was obtained from healthy, male and female, drug-free, human volunteers in accordance with the local research ethics committee at the University of Bristol. A signed consent form in accordance with the Declaration of Helsinki was obtained from each volunteer. Blood was anticoagulated with 4% sodium citrate (1:9) and acidified with acid citrate dextrose (1:7, 25 µg/mL sodium citrate, 20 µg/mL glucose, 15 µg/mL citric acid). Platelet-rich plasma (PRP) was obtained by centrifugation at 180 g for 17 minutes. 10 µM indomethacin and 0.02 U/ml apyrase or 140nM $PGE_1$and 0.02 U/mL apyrase were added to PRP and centrifuged at 520 g for 10 minutes. Platelets were then resuspended at $4x10^8$ cells/mL in modified HEPES-Tyrode's (HT) buffer (145 mM NaCl, 3 mM KCl, 0.5 mM Na2HPO4, 1 mM 5 MgSO4.7H2O, 10 mM HEPES, pH 7.2, 0.1% (w/v) D-glucose, 10 µM indomethacin / 140nM prostaglandin E_1 and 0.02 U/ml apyrase) and rested for 30 minutes at 30°C. All platelet counts were performed using a Z1 Coulter Particle Counter (Beckham Coulter Inc, High Wycombe, UK). Platelets used in all experiments were suspended in HT containing 5.5 mM glucose unless otherwise stated.

Aggregation

Aggregation was induced by PAR1-AP or CRP-XL in washed human platelets ($2x10^8$ cells/mL) and monitored for 5 minutes at 37°C under continuous stirring conditions. Platelets were pre-incubated with either vehicle (HT), glucose or mannitol for 10 minutes prior to agonist stimulation. TPO / IGF-1 were pre-incubated with platelets for 5 minutes before agonist stimulation. Platelets were incubated with metformin for 20 minutes (acute) or 5 hours (chronic) and rosiglitazone for 10 minutes prior to stimulation. All platelet aggregation studies were conducted using a Chrono-log 490 aggregometer (Labmedics, Oxfordshire, UK). Changes in light-transmission were recorded for 5 minutes, from the addition of agonist, using Aggrolink Version 4 software.

Flow cytometry

PS exposure

Annexin V, Alexa Fluor 488 conjugate was used to detect surface PS exposure. Washed human platelets were diluted to $2x10^7$ cells/mL using modified HT (supplemented with 1.2 mM Ca^{2+}). To each well of a 96-well plate 1 µL of Annexin V, 2.5 µL of CRP-XL (0.3-3 µg/mL) and 2.5 µL of Thrombin (0.5-2 M) were added. The diluted platelets were incubated with vehicle (HT) or glucose (10 mins, RT) and vehicle (HT) or rh TPO (5 mins, RT). For experiments including rosiglitazone, platelets were treated with 100 µM rosiglitazone for 10 minutes prior to stimulation. 44 µL of platelets were then added to each well. After 5 minutes

(or 10 minutes depending on desired stimulation time) 200 µL of HT (+ 2mM $CaCl_2$) was added to each well in order to quench the reaction. The plate was then read on a BD Acuri C6 Plus (BD Biosciences, 5000 platelet sample events were recorded, and data was analysed using Flow Version 1.6 software.

Mitochondrial Membrane Depolarisation with TMRM

Tetramethylrhodamine (TMRM) was used as a mitochondrial membrane potential indicator. Washed human platelets ($2x10^8$ cells/mL) suspended in modified HT in the presence of $CaCl_2$ (1.2 mM) were dye loaded with 0.5 µM TMRM (30 mins, RT, in the dark). Platelets were then diluted ($2x10^7$ cells/mL) in modified HT and incubated with either vehicle (HT) or glucose (10 mins, RT) and vehicle (HT) or TPO (5 mins, RT). For experiments including rosiglitazone and metformin, platelets were treated with 100 µM rosiglitazone (10 minutes, 30ºC) or 40 µM metformin (20 minutes, 30°C) prior to stimulation. And then stimulated with both CRP-XL (5 µg/mL) and Thrombin (1U/mL) for 5 minutes. 50 µL of platelets were then added to 150 µL of modified HT in a 96-well plate to quench the reaction. The plate was then read on a BD Acuri C6 Plus. 10,000 platelet sample events were recorded, and data was analysed using Flow Version 1.6 software.

Reactive Oxygen Species Generation with CM-H2DCFDA

CM-H2DCFDA was used as an indicator of ROS generation. Washed human platelets ($2x10^8$ cells/mL) were loaded with CM-H2DCFDA (5 µM, 30mins, 37°C, in the dark) in modified HT in the presence of $CaCl_2$ (1.2 mM). Platelets were then diluted ($2x10^7$ cells/mL) in modified HT in the presence of $CaCl_2$ (1.2 mM) and loaded with CM-H2DCFDA (5 µM) prior to incubation with 5.5mM glucose or 25 mM glucose (10 mins, 37 °C) and vehicle (HT) or 100 ng/mL TPO (5 mins, 37 °C). For experiments including rosiglitazone and metformin, platelets were treated with 100 µM rosiglitazone (10 minutes, 30ºC) or 40 µM metformin (20 minutes, 30°C) prior to stimulation. Samples were then stimulated with 5 µg/mL CRP-XL and increasing concentrations (0.01 - 3 U/mL) of thrombin (5 mins, 37 °C) and the reaction was quenched using dye-loaded HT. Samples were immediately read on a BD Acuri C6 Plus. 10, 000 platelet sample events were recorded, and data was analysed using Flow Version 1.6 software.

P-selectin Exposure and Integrin Activation

Platelet integrin $\alpha_{IIb}\beta_3$ activation and α-granule secretion was monitored by flow cytometry using FITC-conjugated PAC1 antibody (used to assess integrin $\alpha_{IIb}\beta_3$ activation) and PE conjugated CD62P (used to quantify P-selectin exposure as an indicator of α granule secretion). Platelets ($2x10^7$ cells/mL) suspended in modified HT were pre-incubated with either vehicle (H_2O) or 40 µM metformin (20 minutes, 30°C) or vehicle (DMSO or 100

μM rosiglitazone (10 minutes, 30°C). Platelets were then stimulated with 5 μM PAR1-AP or 5 μg/mL CRP-XL for 10 minutes in the presence of FITC PAC1 and PE CD62P. Addition of 2% PFA was used to fix the reaction. Samples were analysed on a BD Accuri C6 Plus (BD Bioscience) using FACS Diva software (BD Bioscience, Oxford, UK).

Calcium signalling

PRP, prepared as previously stated, was loaded with 4 μM Fura2 AM (1 hr, 30°C). Platelets were collected by centrifugation at 520 g for 10 minutes and re-suspended in modified HT at $2x10^8$ cells/mL and rested for 30 minutes to allow complete de-esterification of intracellular AM esters (the de-esterification process liberates the Ca^{2+}-sensitive indicator). Fluorescence was monitored and recorded at 37°C using Tecan infinite M200 Pro multimode reader (Tecan Trading AG, Switzerland) and excitation at 340 nm and 380 nm before and after addition of agonist. 20% Triton was added to each well to cause cells to lyse and maximum Ca^{2+} release, followed by addition of 0.5 M EGTA and 0.5 M TRIS at pH 7.4 used to chelate divalent cations and provide a minimum Ca^{2+} release recording. Changes in cytosolic [Ca^{2+}] were monitored using the 340/380 nm fluorescence ratio.

Data analysis

All data were analysed and curves fitted using GraphPad Prism 7.02 software (GraphPad Software, San Diego, CA, USA). Data are presented as the mean ± standard error of the mean (SEM) of a minimum of three independent experiments. Data that has been presented with statistical analysis were tested using either a two-tailed paired Student's t-test, a two-way ANOVA with a Bonferroni multiple comparison post hoc test or a one-way ANOVA with a Dunnett's multiple comparisons post hoc test. A students T-test was used to test the hypothesis: there is a difference between two sample means. A one-way ANOVA with Dunnett's multiple comparison post hoc test was used to compare each of a number of treatments to a single control group. And a two-way ANOVA with Bonferroni multiple comparison post hoc was used to compare concentration response curves. For some of the statistical analysis I had to assume the data was normally distributed. A P value of less than 0.05 was considered statistically significant: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

Results

Acute hyperglycaemia does not alter platelet aggregation

In order to determine whether hyperglycaemia can contribute to platelet hyperreactivity in diabetics, the effect of high/increased glucose concentrations on platelets from healthy volunteers was investigated. Washed human platelets in the presence of either 5.5 mM or 25 mM glucose or 5.5 mM glucose + 19.5 mM mannitol were stimulated with either PAR1-AP or CRP-XL and aggregation continuously monitored (Fig. 5A-D). Mannitol was used as an osmotic control. Mannitol is a sugar alcohol that is not transported into platelets and is commonly used as an osmotic control for high glucose treatment. Pre-incubation of platelets for 10 minutes with 25 mM glucose was observed to not significantly alter platelet aggregation induced by CRP-XL or PAR1-AP compared to 5.5 mM glucose (Fig.5A-D). As the effect of glucose may be time dependent, the experiment was repeated using various incubation times with glucose. Platelets were incubated with high glucose or mannitol for 10, 20, 30, 60 and 90 minutes and aggregation continuously monitored (Fig.5E). Extending the time platelets were incubated with 25 mM glucose also failed to significantly alter platelet aggregation. Concentration-response curves can be bell shaped, with molecules inducing differing effects at low and high concentrations. Therefore, to investigate whether a concentration of glucose > 5.5 mM but < 25 mM could alter platelet function, aggregation was recorded using platelets that had been preincubated with a range of glucose concentrations (Fig. 5F). However, none of the glucose concentrations tested significantly altered CRP-XL stimulated platelet aggregation when compared to normal (5.5 mM) glucose.

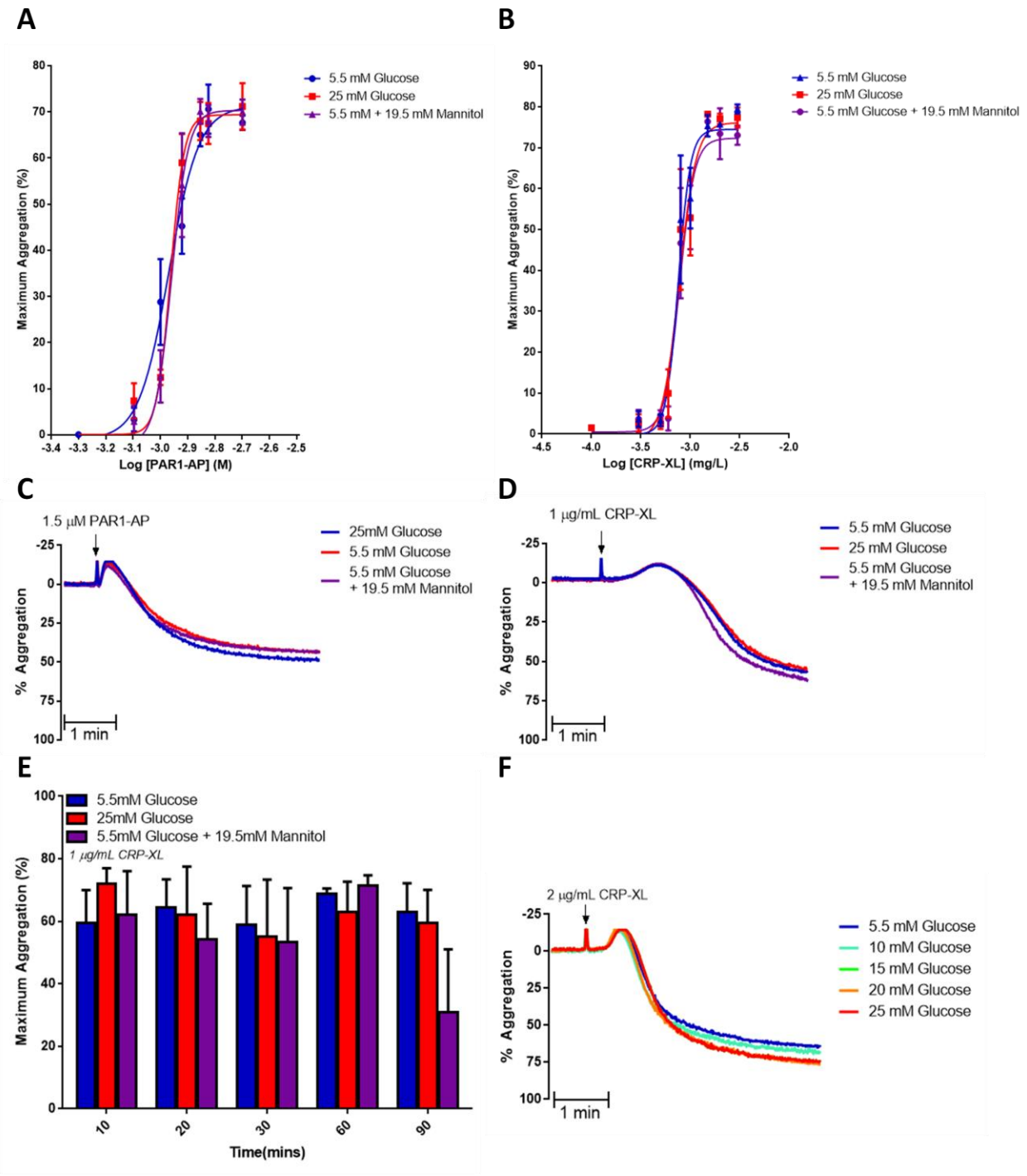

Figure 5: The effect of D-glucose on platelet aggregation. Washed human platelets (2×10^8/mL) were incubated with 5.5 mM glucose, 25 mM glucose or 5.5 mM glucose + 19.5 mM mannitol for 10 minutes before stimulation. **(A)** Platelets were stimulated with increasing concentrations of CRP-XL (0.1- 3 µg/mL) and maximum aggregation was recorded. NS. N=8. A two-way ANOVA with a Bonferroni multiple comparisons post-hoc test was performed. **(B)** Representative aggregation trace. **(C)** Platelets were stimulated with increasing concentrations of PAR1-AP (0.5-2 µM) and maximum aggregation was recorded. NS. N=3. A two-way ANOVA with a Bonferroni multiple comparisons post-hoc test was performed **(D)** Representative aggregation trace **(E)** A bar chart showing the effect of platelet incubation time with glucose/ mannitol on the effects of 25 mM glucose and 19.5 mM mannitol on CRP-XL (1 µg/mL) platelet aggregation compared to 5.5 mM glucose. NS. N=4. A two-way ANOVA test was performed. **(F)** A representative aggregation trace showing the effects of different glucose concentrations on maximum aggregation.

TPO-mediated enhancement of aggregation is unaltered by hyperglycaemia

TPO is a known platelet primer and has been shown to increase platelet aggregation in several publications (Blair, Moore & Hers, 2015). Initially, my aim was to reproduce previous findings by treating platelets with TPO (100 ng/mL, 5 min) prior to platelet stimulation with CRP-XL and demonstrating whether platelet aggregation was enhanced in the presence of TPO. My data confirms that 100 ng/mL TPO significantly enhances CRP-XL mediated platelet aggregation (Fig 6A-B). TPO alone did not induce platelet aggregation (data not shown). IGF-1 is also a known platelet primer that has been shown to increase platelet aggregation. Figure 6C shows that IGF-1 also significantly enhanced CRP-XL stimulated platelet aggregation. I was also interested in whether higher concentrations of glucose could alter the effect of TPO on enhancing platelet function. Therefore, platelets were pre-incubated with 5.5 or 25 mM glucose in the presence or absence of 100 ng/mL TPO before stimulation with CRP-XL. Incubation of platelets with 25 mM glucose did not alter the ability of TPO to enhance platelet aggregation (Fig. 6D-E).

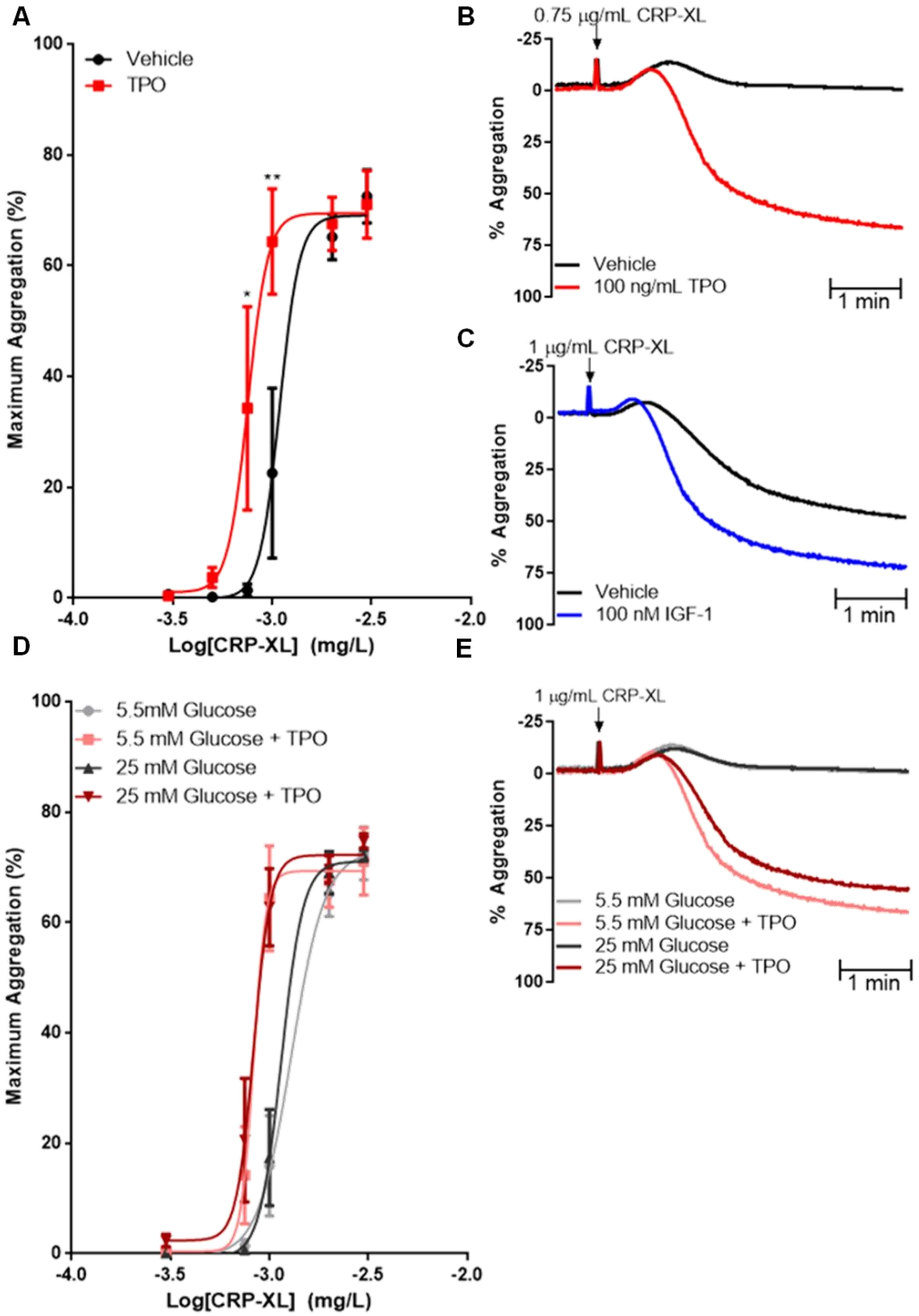

Figure 6: The effect of platelet primers and glucose on aggregation. (A-C) Washed human platelets (2x10^8 cells/ mL) were incubated with 100 ng/mL TPO, 100 nM IGF-1 or vehicle (HT) for 5 minutes prior to stimulation with a sub threshold concentration of CRP-XL (0.7-1 µg/mL). **(A)** Concentration-response curve showing the effects of TPO on maximum platelet aggregation. N=3. **(B and C)** Representative aggregation traces. **(D-E)** Washed human platelets (2x10^8 cells/ mL) were incubated with normal (5.5 mM) or high (25 mM) glucose for 10 minutes. Platelets were also treated with either vehicle (HT) or 100 ng/mL TPO for 5 minutes prior to stimulation. **(D)** Concentration-response curve showing the effect of TPO, glucose and TPO+glucose on aggregation. N=3. NS. **(E)** Representative aggregation trace. Two-way ANOVA with a Bonferroni multiple comparisons post-hoc test was performed.

TPO enhances platelet PS exposure

PS exposure is another important marker of platelet activation as it is fundamental for thrombin generation and therefore plays a key role in the blood coagulation process. It is important to also consider the effect glucose and TPO on this aspect of platelet function. In resting platelets PS is found on the inner leaflet of the plasma membrane but under certain conditions (dual stimulation, apoptosis) it is flipped to the outer leaflet by an enzyme called scramblase and can be probed using the PS-binding protein annexin V. Initially, experiments were set up to determine the optimum conditions for TPO to enhance PS exposure (Fig. 7A-B). Some preliminary experiments, investigating the effect of TPO on PS exposure when platelets were stimulated with both CRP-XL and thrombin for a range of stimulation times (2 - 30 minutes), indicated that TPO had its greatest effect at the lower stimulation time points (data not shown). Therefore the effect of TPO on PS exposure when platelets were stimulated for 5 and 10 minutes was investigated in more detail. Pre-treatment of platelets with TPO for 5 minutes followed by stimulation with agonist (CRP-XL and thrombin) for 10 minutes demonstrated that TPO under these conditions did not significantly alter PS exposure (Fig. 7A). However, platelets pre-treated with TPO for 5 minutes followed by stimulation with agonist (CRP-XL and thrombin) for 5 minutes demonstrated TPO could significantly enhance PS exposure (Fig. 7B).

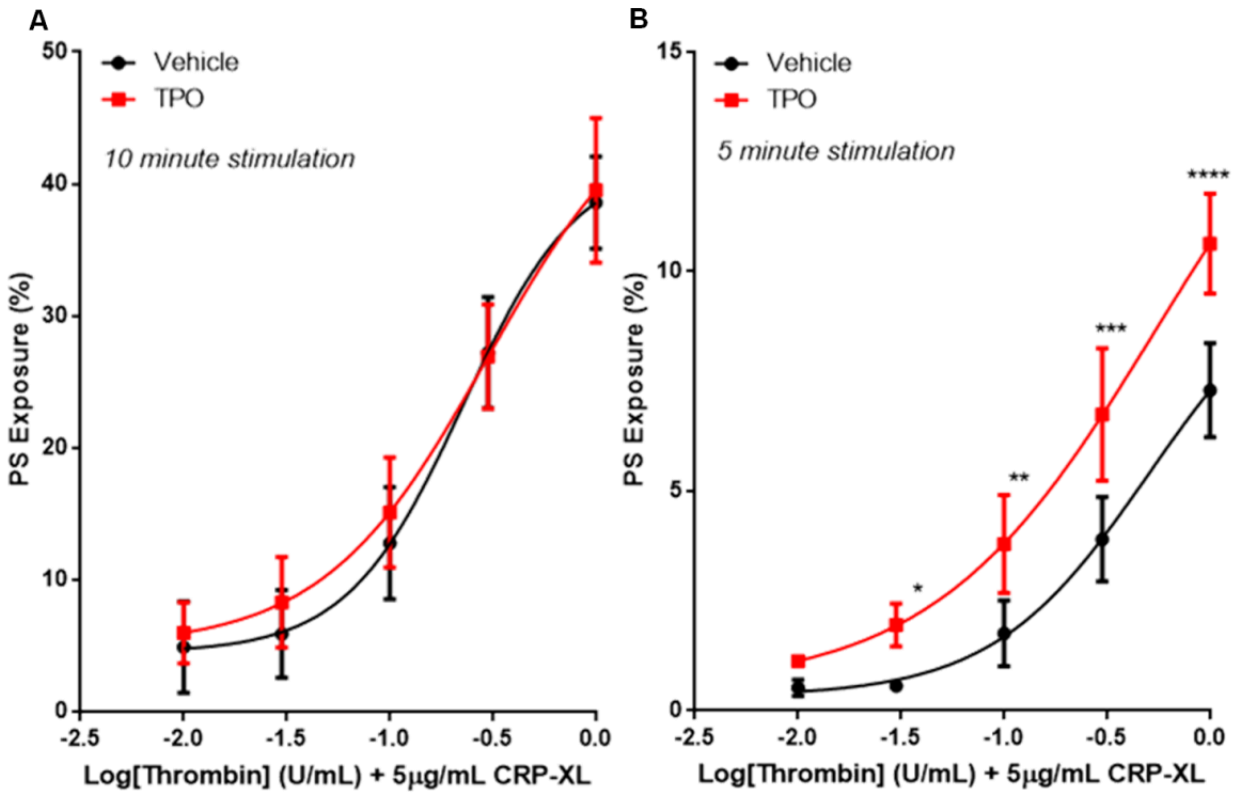

Figure 7: TPO enhances agonist-mediated PS exposure. Washed human platelets ($2x10^7$ cells/mL) were treated with either 100 ng/mL TPO or vehicle (HT) for 5 minutes prior to stimulation with 5 µg/mL CRP-XL and increasing concentrations of thrombin (0.01 - 1 U/mL). Percentage of platelets exposing PS was recorded as a result of CRP-XL and thrombin stimulation for **(A)** 10 minutes (NS) and **(B)** 5 minutes. N=3. Two-way analysis of variance with Bonferroni multiple comparisons post hoc test was performed.

Hyperglycaemia enhances platelet PS exposure

Next, the effect of hyperglycaemia and the combined effect of hyperglycaemia and TPO on PS exposure was assessed. Figure 8A shows that platelets pre-treated with 25 mM glucose for 10 minutes prior to agonist stimulation exposed significantly more PS compared to platelets pre-treated with the normal 5.5 mM glucose. The combined pre-treatment of high glucose and TPO (Fig. 8C) had no additive effect compared to glucose and TPO alone (Fig. 8A and B, respectively). I then tested the effect of a range of glucose concentrations (5.5, 10, 15, 20 and 25 mM) on agonist (CRP-XL and thrombin) mediated PS exposure. Figure 8D shows the EC50 values of the concentration response curves decreases as the concentration of glucose increases. This shows that glucose concentration dependently enhances platelet PS exposure.

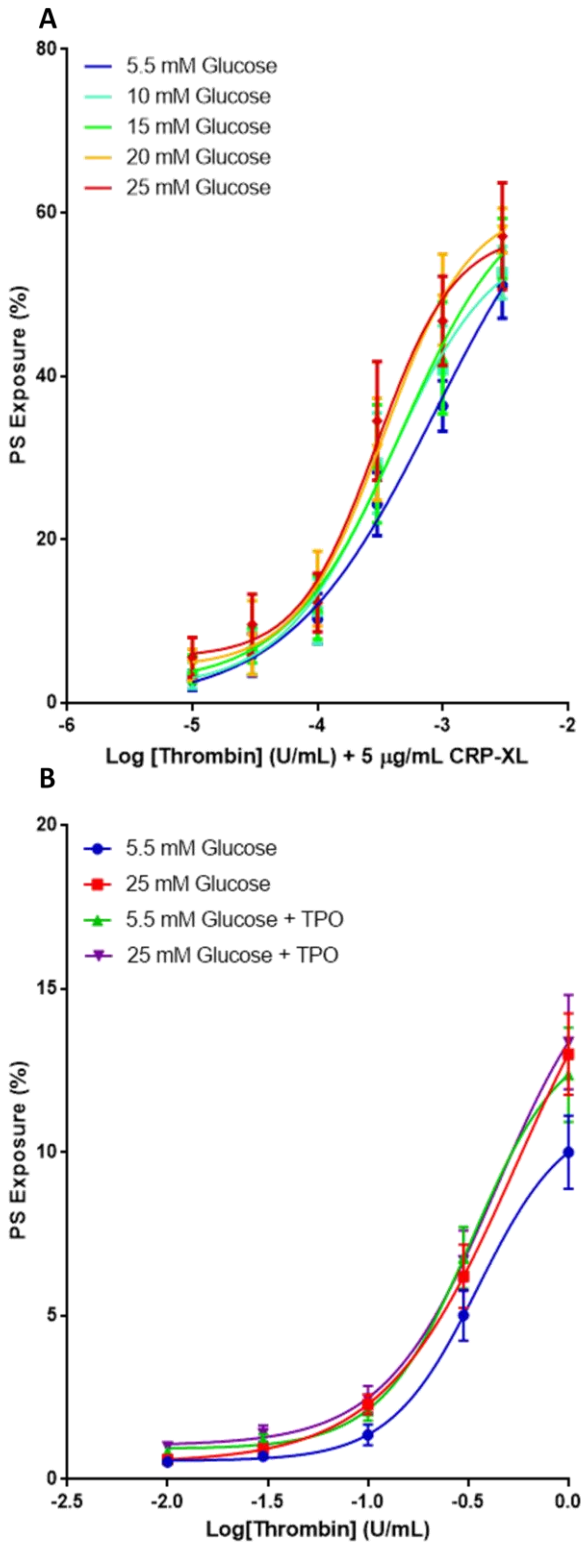

Figure 8: Glucose enhances agonist-mediated PS exposure. (A) Washed human platelets ($2x10^8$ cells/mL) were incubated with 5.5 mM glucose or 25 mM glucose for 10 minutes and 100 ng/mL TPO or vehicle (HT) for 5 minutes prior to stimulation with 5 µg/mL CRP-XL and increasing concentrations of thrombin (0.01-1 U/mL) and PS exposure was recorded. N=6. **(B)** Platelets, incubated with various concentrations of glucose for 10 minutes, were stimulated with 5 µg/mL CRP-XL and increasing concentrations of thrombin (0.01-1 U/mL) for 5 minutes and PS exposure was recorded.

TPO and hyperglycaemia alter agonist-mediated changes in ROS generation and mitochondrial membrane potential

As previously mentioned, both platelet ROS generation and mitochondrial membrane depolarisation have been linked to an increase in PS exposure. I therefore explored the effect of TPO and hyperglycaemia on ROS generation and mitochondrial membrane depolarisation in order to validate the observed effect both TPO and glucose had on PS exposure. Initially, I investigated the effect of TPO and high glucose on the ability of platelets to produce ROS using the CM-DCFDA dye. Non-fluorescent CM-H2DCFDA passively diffuses into platelets and upon oxidation by ROS it becomes highly fluorescent and this fluorescence can be recorded using a flow cytometer. Figure 9A shows that platelets pre-treated with 100 ng/mL TPO for 5 minutes generated significantly more ROS when compared to platelets pre-treated with vehicle. Interestingly, platelets pre-incubated with high glucose for 10 minutes generated the same amount of ROS as platelets pre-incubated with normal glucose (Fig. 9B). And figure 9C shows that hyperglycaemia does not affect the ability of TPO to enhance platelet ROS generation.

I then used TMRM to detect any change in agonist-mediated mitochondrial membrane potential due to the presence of TPO or high glucose. TMRM is a cell-permeable dye that accumulates in active mitochondria. The fluorescent signal that TMRM produces can be directly related to a change in mitochondrial membrane potential ($\Delta\Psi m$); upon membrane depolarisation TMRM leaks from within the mitochondria leading to a reduction in the intensity of the fluorescence. The TMRM signal can be detected and recorded using flow cytometry. Figure 9D shows the effect of platelet pre-treatment with TPO, 25 mM glucose and TPO + 25 mM glucose on stimulated platelet mitochondrial membrane potential compared to platelets pre-treated with 5.5 mM glucose. In accordance with their effect on platelet PS exposure both TPO and hyperglycaemia enhance mitochondrial membrane depolarisation but there is no additive effect when platelets are pre-treated with both 25 mM glucose and 100 ng/mL TPO (Fig. 9D).

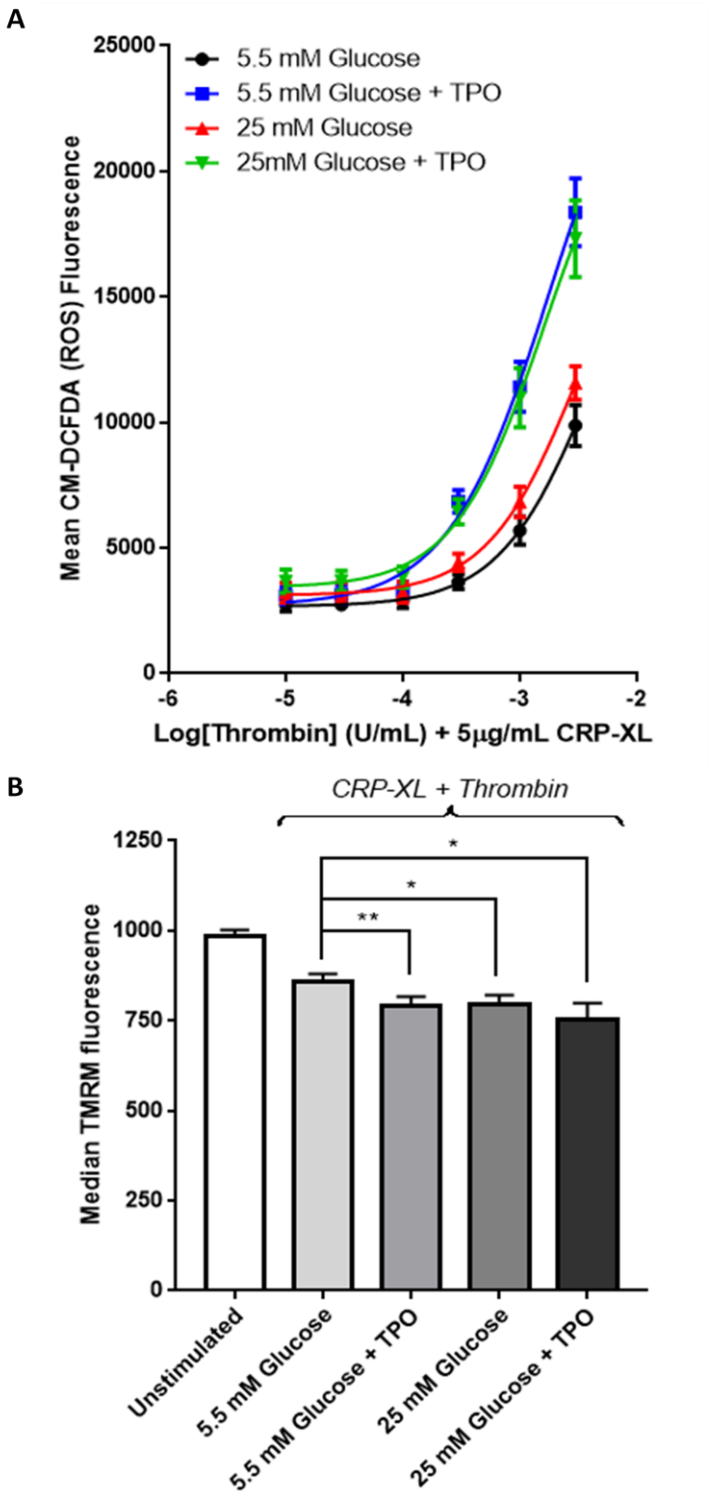

Figure 9: The effect of glucose and TPO on ROS generation and ΔΨm. Washed human platelets ($2x10^7$ cells/mL) were incubated with 5.5 mM glucose or 25 mM glucose for 10 minutes and vehicle (HT) or 100 ng/mL TPO for 5 minutes prior to stimulation with 5 µg/mL CRP-XL and increasing concentration of thrombin (0.01-3 U/mL). **(A)** A concentration response curve showing the effects of hyperglycaemia, TPO, and hyperglycaemia and TPO on mean CM-DCFDA fluorescence. N=5. **(D)** The effects of 10 minute incubation with 25 mM glucose or 5.5 mM glucose and 5 minute treatment with 100 ng/mL TPO or vehicle (HT) prior to stimulation with 5 µg/mL CRP-XL and 1 U/mL thrombin on washed human platelets ($2x10^7$ cells/mL) on mitochondrial membrane potential were recorded as the median TMRM fluorescence. N=8. One-way ANOVA with a Dunnett's post hoc test was performed.

Hyperglycaemia reduces agonist-mediated increases in intracellular calcium

Another mechanism which has been shown to be involved in enhanced PS exposure is an increase in calcium mobilisation. I therefore also investigated the effects of 100 ng/mL TPO and 25 mM glucose on platelet calcium mobilisation using Fura-2 AM. Fura-2 AM is a membrane-permeable calcium indicator and once inside the platelet and bound to Ca^{2+} it fluoresces. Intracellular calcium concentrations in washed platelets were increased following stimulation with 5 µg/mL CRP-XL and increasing concentrations of thrombin (0.01 - 3 U/mL) (Fig. 10). Pre-treating the platelets with TPO for 5 minutes prior to stimulation did not alter calcium mobilisation compared to vehicle (Fig. 10A-B). In contrast, when the platelets were pre-incubated with 25 mM glucose for 10 minutes this led to a significant decrease in calcium signalling (Fig. 10C-D), which was reversed in the presence of TPO (Fig. 10E-F). This experiment was repeated with the osmotic control, mannitol, to ensure that the reduction in calcium signalling seen with high glucose wasn't due to an increase in osmolarity leading to Fura-2 AM dye leaking out of the cell. Figure 10G and 10H shows that treating washed human platelets with 19.5 mM mannitol had no effect on platelet calcium signalling in comparison to vehicle. Together, these results show that glucose significantly reduced intracellular calcium mobilisation.

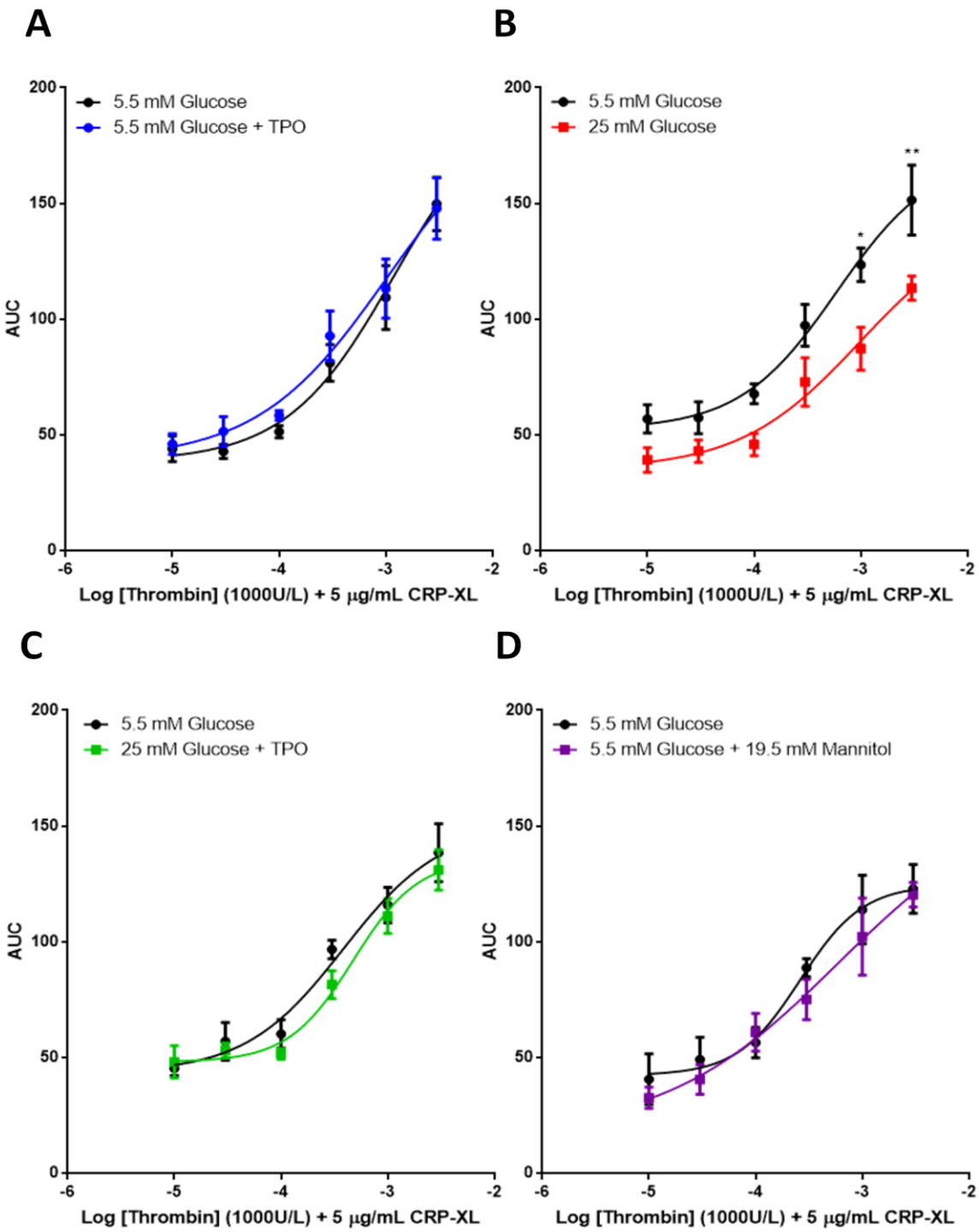

Figure 10: The effect of glucose and TPO on intracellular calcium signalling. Washed human platelets were incubated with normal glucose, high glucose or mannitol for 10 minutes and vehicle (HT) or 100 ng/mL TPO for 5 minutes prior to stimulation with 5 µg/mL CRP-XL and increasing concentrations of thrombin. **(A-D)** Dose-response curves, represented as area under the curve (AUC), showing the effects of 25 mM glucose, 100 ng/mL TPO, 25 mM glucose + 100 ng/mL TPO and 5.5 mM glucose + 19.5 mM mannitol, respectively, on calcium mobilisation compared to vehicle. N=6. A two-way ANOVA with Bonferroni post hoc test was performed.

Inhibition of platelet function by aspirin is not altered by the presence of glucose

Aspirin is a “gold-standard” anti-platelet drug and has been shown to decrease platelet function. It has been suggested that high blood glucose levels can reverse the inhibition of platelet function induced by aspirin (aspirin-resistance) (Kobzar, Mardla & Samel, 2011). Treatment of platelets with 30 mM aspirin for 10 minutes significantly reduces CRP-XL stimulated aggregation (Fig.11A), whereas aspirin had no significant effect on PAR1-AP stimulated aggregation (Fig. 11B). Incubating platelets in the presence of 25 mM glucose was observed to reduce the effect of aspirin on CRP-mediated aggregation, however this reduction did not reach significance and was highly variable between donors (Fig.11A). As previously mentioned, hyperglycaemia has no effect on aggregation but I have shown that it is able to enhance PS exposure. I therefore decided to investigate the effect of aspirin on PS exposure and whether or not the presence of a high concentration of glucose would be able to reverse any effect of aspirin. Interestingly, platelet treatment with aspirin had no effect on platelet PS exposure (Fig.11C). P2Y12 blockers are anti-platelet drugs that are frequently used in the clinic. ARC is a P2Y12 antagonist that is able to reduce PS exposure (Fig. 11C), however, this inhibition could not be rescued by pre-treatment with TPO, 25 mM glucose or TPO+ high glucose (Fig. 11C).

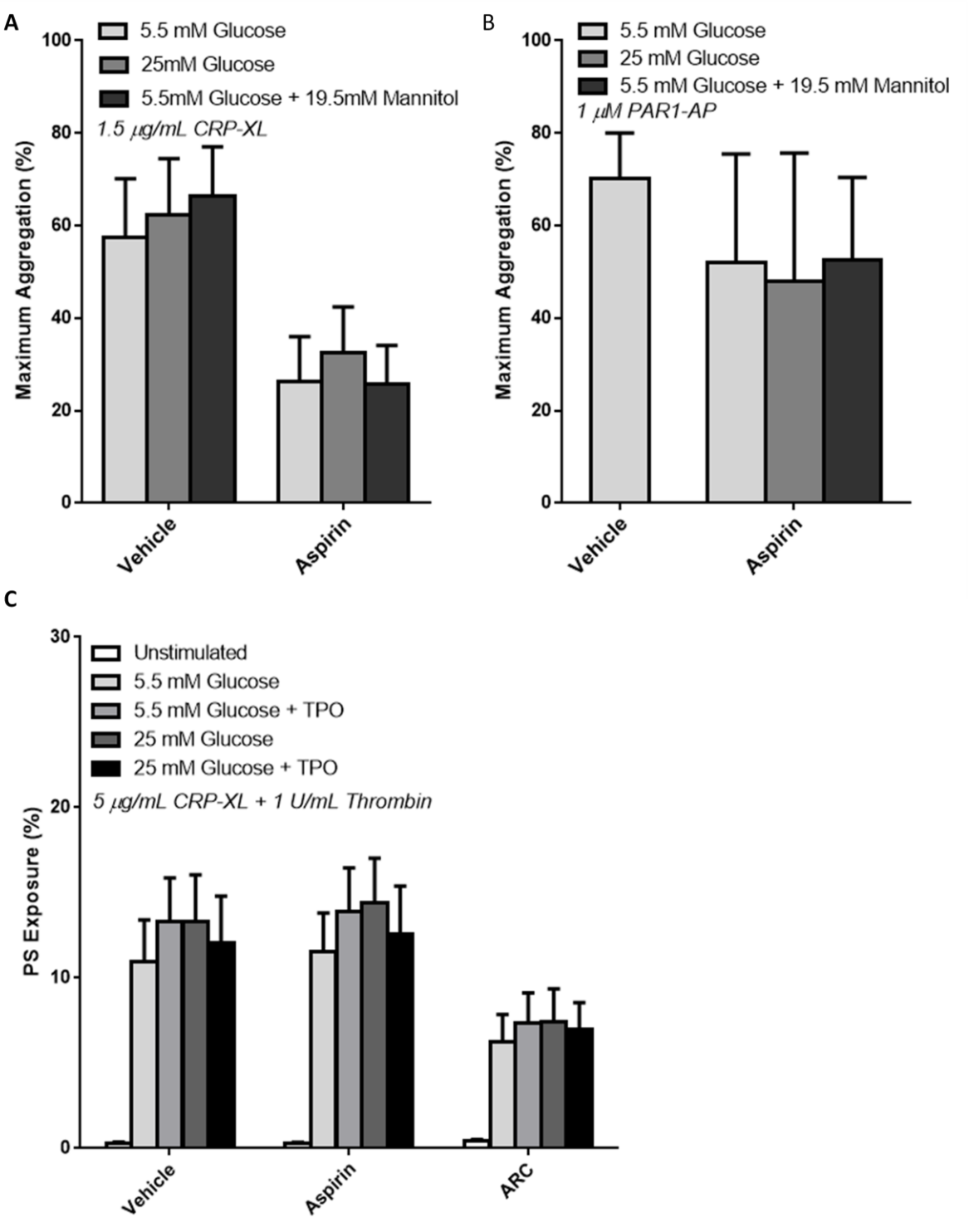

Figure 11: Hyperglycaemia has no effect on the inhibition of platelet function by aspirin. Platelets treated with 30 mM aspirin for 10 minutes showed reduced aggregation and the effects of high glucose and mannitol on this affect were recorded in response to **(A)** 1.5 µg/Ml CRP-XL stimulation. N=5. And **(B)** 1 µM PAR1-AP stimulation. N=2. **(C)** A bar chart showing the effect of 25 mM glucose, 100 ng/mL TPO and 25 mM glucose+100ng/mL TPO on the effect of aspirin and ARC treatment on platelet PS exposure. N=6.

The anti-diabetic therapy metformin does not alter platelet function.

Metformin is the most commonly prescribed anti-diabetic treatment and it has been suggested that metformin can also act as an anti-platelet therapy (Xin et al., 2016). Initially, I investigated the effects of 40 μM metformin on CRP-XL and PAR1-AP stimulated platelet aggregation. In T2DM patient treated with metformin, systemic plasma concentration of metformin is likely to be 10 - 40 μM (He & Wondisford, 2015). Therefore, for these experiments I chose to use a final metformin concentration of 40 μM as this represents a therapeutic concentration found in patients with T2DM. Washed human platelets ($2x10^8$ cells/mL) were incubated with metformin for 20 minutes prior to stimulation and aggregation was monitored and maximum aggregation reached was recorded. Metformin was observed to reduce platelet aggregation however this did not reach significance (Fig. 12A, C). Chronic incubation of platelet with metformin (5 hours in platelet-rich plasma) was also observed to reduce aggregation, but again this did not reach significance (Fig. 12B, D). Furthermore, treatment with metformin had no effect on CRP-XL or PAR1-AP mediated platelet integrin $\alpha_{IIb}\beta_3$ activation and p-selectin expression compared to agonist alone (Fig.12 E- F).

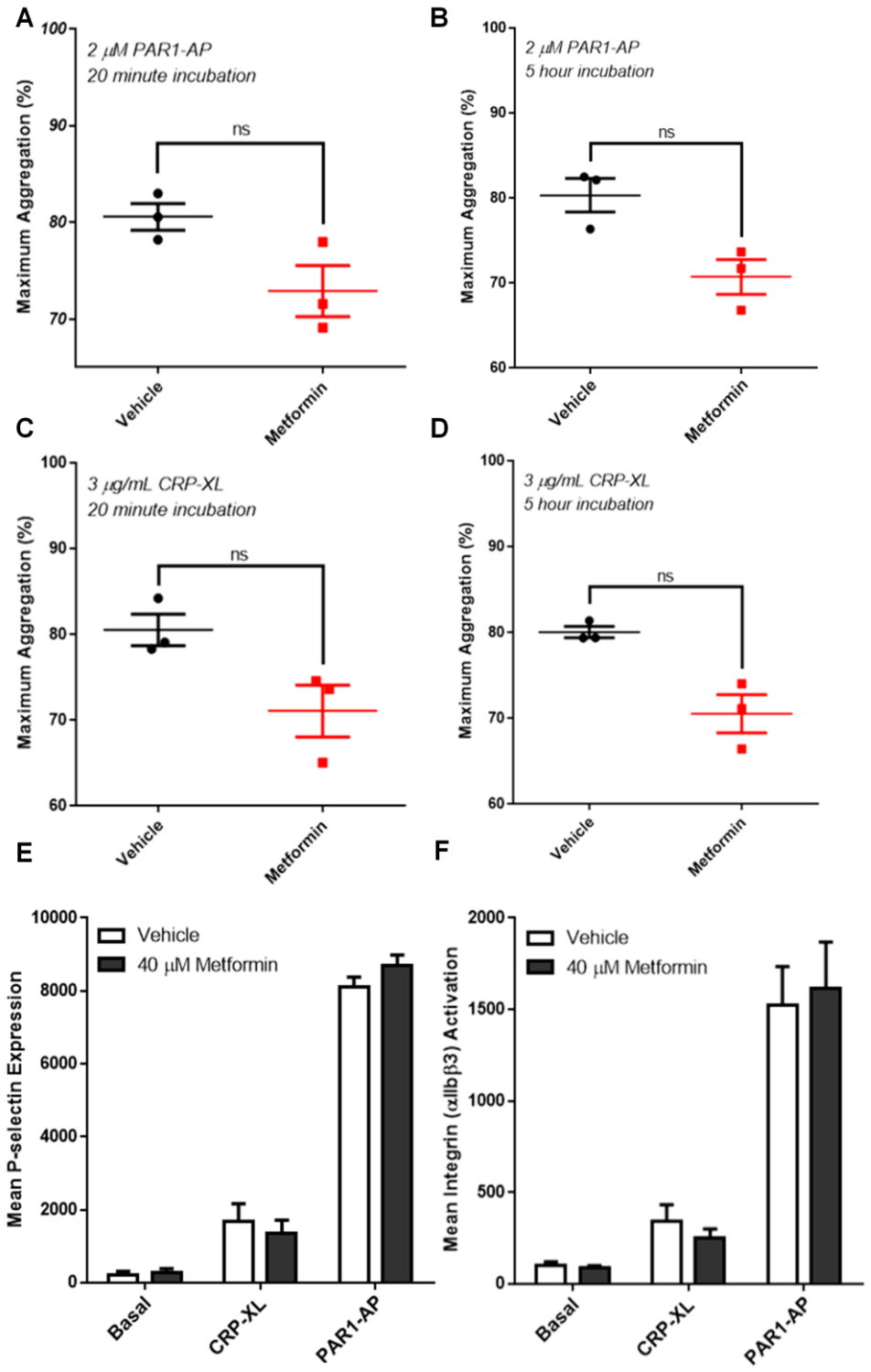

Figure 12: The effect of Metformin on platelet priming and function. (A+C) Washed human platelets ($2x10^8$ cells/mL) were incubated with 40 μM metformin or vehicle (DMSO) for 20 minutes (acute) prior to stimulation with either PAR1-AP or CRP-XL and the maximum aggregation reached over a 5 minute stimulation was recorded. N=3. Paired students t-test was performed **(B+D)** PRP was incubated with 40 μM metformin for 5 hours (chronic) prior to PAR1-AP or CRP-XL stimulation, maximum aggregation was recorded. A paired students t-test was performed. The effect of acute metformin treatment on washed human platelets ($2x10^7$ cells/mL) **(E)** alpha granule secretion and **(F)** integrin (αIIbβ3) activation was recorded over a 10 minute stimulation period with either 5 μM PAR1-AP or 5 μg/mL CRP-XL. N=3.

The anti-diabetic therapy rosiglitazone inhibits platelet aggregation but enhances ROS generation and PS exposure.

Rosiglitazone is another anti-diabetic drug that has been suggested to have anti-platelet properties (Liu, Park, Chang, Huh, Lee & Lee, 2016) and to investigate this further, the effect of 100 μM rosiglitazone on CRP-XL and PAR1-AP stimulated platelet aggregation was evaluated. Figure 13 A,B,D and E shows that platelet pre-treatment with rosiglitazone for 10 minutes prior to stimulation results in a statistically significant reduction in both CRP-XL and PAR1-AP stimulated maximum aggregation. Figure 13C and 13F shows that 100 ng/mL TPO is unable to rescue the inhibitory effects of rosiglitazone on both CRP-XL and PAR1-AP stimulated platelet aggregation. Due to the significant effect of rosiglitazone on platelet aggregation, I aimed to further investigate the effects of rosiglitazone on platelet function. Figure 14 shows that pre-treatment with rosiglitazone for 10 minutes prior to stimulation slightly reduced both CRP-XL and PAR1-AP stimulated platelet integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression in comparison to vehicle but this did not reach significance.

Next, I studied the effect of rosiglitazone on PS exposure and, unexpectedly, I found that platelets treated with rosiglitazone exposed significantly more PS when stimulated with CRP-XL and thrombin for 5 minutes compared to vehicle. Pre-treatment with either 100 ng/mL TPO or 25 mM glucose as well as 100 μM rosiglitazone had no additive or inhibitory effect on the enhancement of PS exposure seen with rosiglitazone alone (Fig. 15A+B). I subsequently investigated the effect of rosiglitazone on platelet ROS generation and mitochondrial membrane potential. As treatment with rosiglitazone resulted in an increase in PS exposure, I hypothesised that it will also cause an increase in ROS generation and mitochondrial membrane depolarisation. As predicted figure 15C shows that pre-treatment with rosiglitazone caused an increase in platelet ROS generation compared to vehicle but not to the same extent as TPO alone. Pre-treatment with the combination of both rosiglitazone and TPO significantly enhanced platelet ROS generation and pre-treatment with high glucose appears to reduce the enhanced ROS generation caused by the presence of rosiglitazone (Fig. 14C). I then considered the effect of rosiglitazone on platelet mitochondrial membrane potential. Unexpectedly, pre-treatment with rosiglitazone actually significantly reduced CRP-XL and thrombin stimulated platelet mitochondrial membrane depolarisation in comparison to vehicle (Fig. 14D). Pre-treatment with either 100 ng/mL TPO or 25 mM glucose does not appear to affect the inhibition of platelet

mitochondrial membrane depolarisation seen in the presence of rosiglitazone (Fig. 14D).

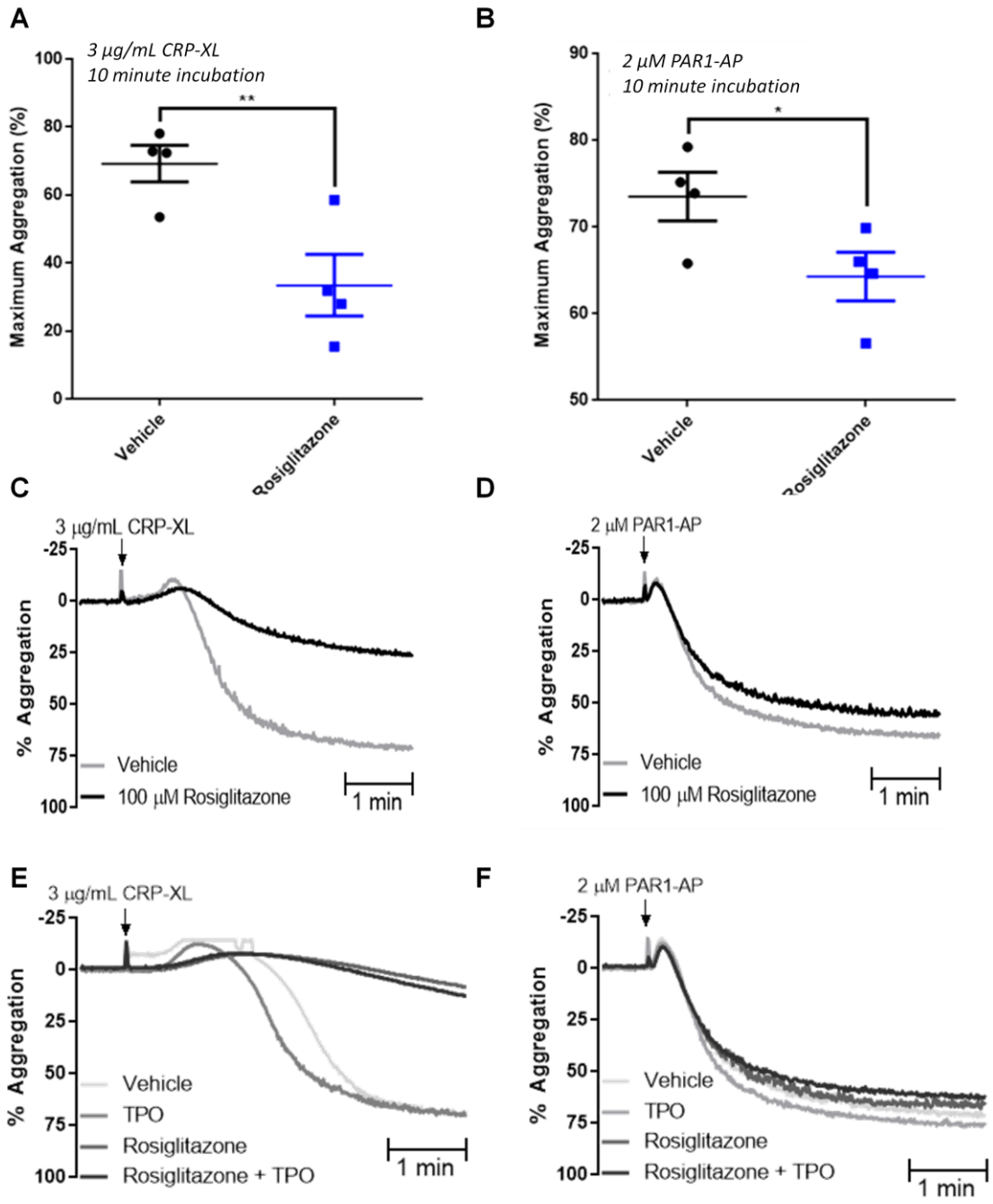

Figure 13: Rosiglitazone inhibits platelet aggregation. The effect of 100 µM rosiglitazone (10 minute incubation) on washed human platelet ($2x10^8$ cells/mL) maximum aggregation, stimulated with **(A)** 3 µg/mL CRP-XL and **(B)** 2 µM PAR1-AP. N=4. Paired students t-test was performed. **(C+D)** Representative aggregation traces. **(E+F)** Representative aggregation traces showing the effect on 3 µg/mL CRP-XL or 2 µM PAR1-AP stimulated platelet aggregation of pre-treatment with 100 µM rosiglitazone or vehicle (DMS0) for 10 minutes and either vehicle (HT) or 100 ng/mL TPO for 5 minutes.

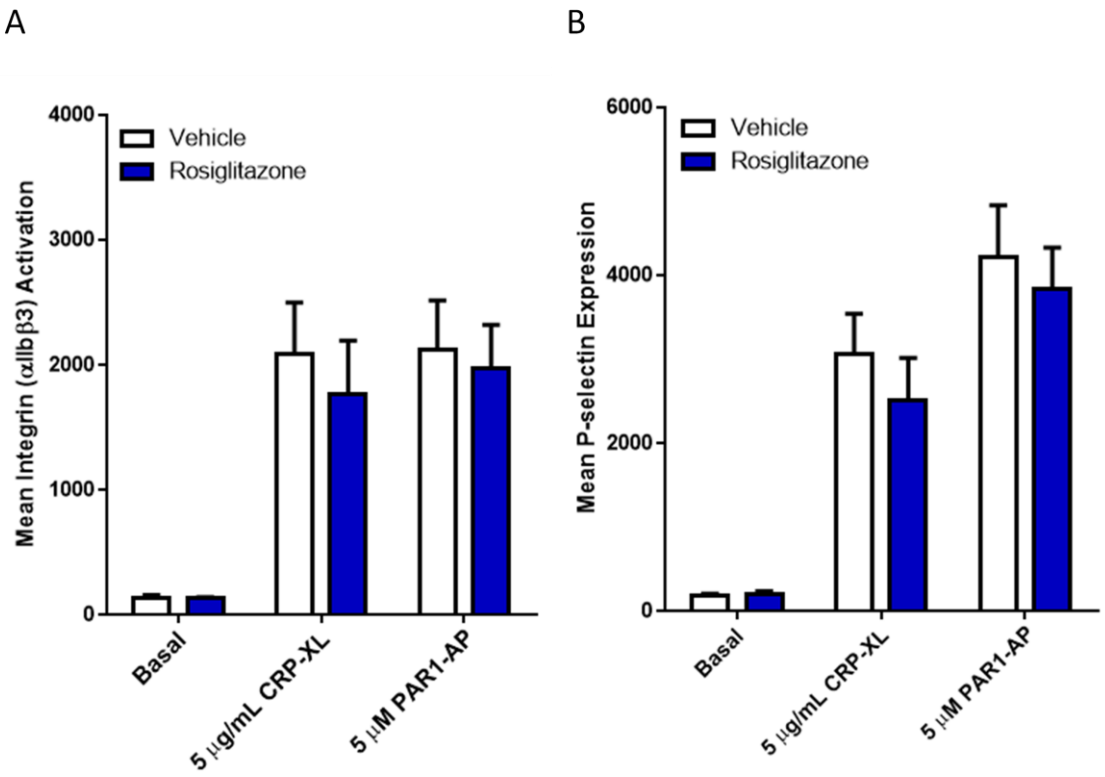

Figure 14: The effect of Rosiglitazone on P Selectin expression and Integrin actication. Washed human platelets ($2x10^7$ cells/mL) were treated with 100 μM rosiglitazone and the effect on CRP-XL and PAR1-AP stimulated **(C)** p-selectin expression and **(D)** integrin activation compared to vehicle (DMSO) were recorded after 10 minutes of stimulation. N=3. **(E)**The effect of the different conditions on 3 μg/mL CRP-XL and 1 U/mL thrombin stimulated ROS generation. N=6

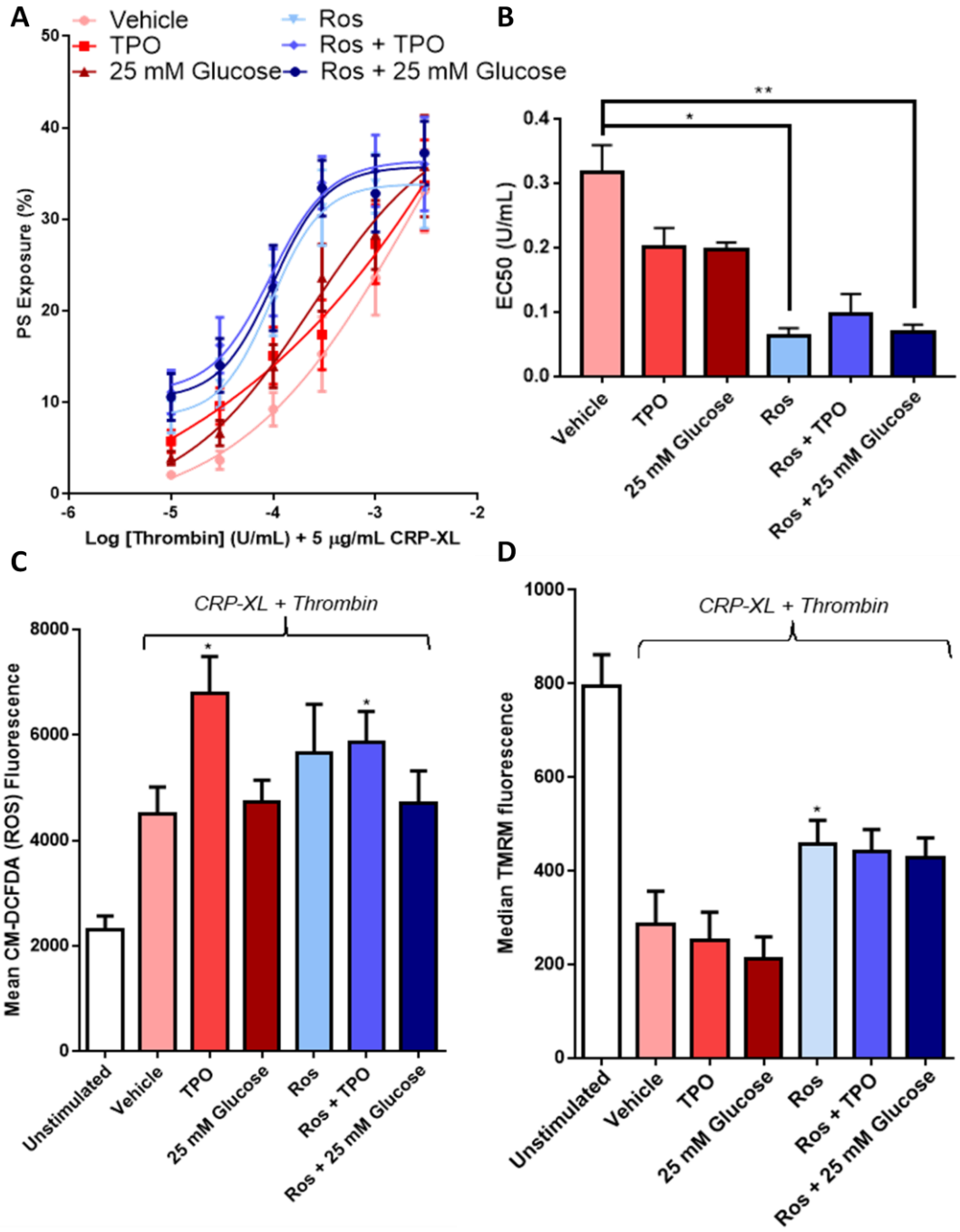

Figure 15: The effect of Rosiglitazone on platelet priming and function. (A) Washed human platelets were stimulated with 5 µg/mL CRP-XL and thrombin (0.01 - 3 U/mL) for 5 minutes. The percentage of platelets expressing PS was recorded for each concentration of thrombin and plotted as a dose-response curve. N=6. **(B)** The EC50 value for each condition. N=6. A one-way ANOVA with a Dunnett's post hoc test was performed. . **(F)**The effect of TPO, high glucose and rosiglitazone on 3 µg/mL CRP-XL and 1 U/mL thrombin stimulated mitochondrial membrane potential over a 5 minute stimulation period. N=6. Platelets were incubated with either 5.5 mM glucose or 25 mM glucose for 10 minutes, 100 µM rosiglitazone or vehicle (DMSO) for 10 minutes and vehicle (HT) or 100 ng/mL TPO for 5 minutes prior to being stimulated. A one-way ANOVA with a Dunnett's post hoc test was performed.

Discussion

Platelet hyperreactivity is a known cause of increased CVD risk and understanding the mechanisms behind this enhanced reactivity is fundamental to finding a solution and reducing CVD risk. This study aimed to investigate the cause of platelet hyperreactivity seen in diseases such as DM. The initial investigation into the effects of hyperglycaemia on platelets showed that high glucose concentrations enhanced a number of platelet functions, including PS exposure, but had no effect on agonist-mediated aggregation. I was also able to further add to the literature regarding the ability of TPO to enhance platelet activity. Interestingly, this study has demonstrated that there is no cumulative effect seen when platelets are treated with TPO in combination with high glucose. The final aim of this study was to explore the idea that anti-diabetic treatments are able to reverse hyperreactive qualities induced by TPO and/or hyperglycaemia.

Hyperglycaemia has no effect on platelet aggregation but enhances PS exposure

This study has shown that for CRP-XL or PAR1-AP stimulated platelet aggregation there is no significant difference between platelets that were pre-incubated with a high glucose concentration (25 mM) or a normal glucose concentration (5.5 mM) (Fig. 5). This data contradicts the research done by Tang et al who showed that collagen stimulated aggregation was enhanced when platelets were pre incubated with high glucose (Tang et al., 2011) and other studies with similar results (table 2). There have been several studies that also demonstrated that high glucose alone has no effect on platelet aggregation (Kobzar, Mardla & Samel, 2011; Kobzar, Mardla & Samel, 2017). The reason for such large variance in the literature is most likely down to the vast range of ways in which the platelets are subjected to increased glucose levels. For example, some studies use hyperglycaemic clamps in healthy and diabetic controls where as others simply use very acute hyperglycaemic treatment in washed healthy human platelets. In general, the incubation of platelets with high glucose resulted in large variance in my experiments and I believe this may be due to the wide range of blood glucose levels experienced by different volunteers prior to blood donation. And this is likely to have contributed to some of the variability seen in the present study.

Here, for the first time, I have shown that hyperglycaemia directly results in increased platelet exposure of PS (Fig. 8A) and mitochondrial membrane depolarisation (Fig. 9D). Increased ROS generation has been shown to enhance MPTP formation and PS exposure in platelets. However, it has also been

demonstrated that ROS generation is independent of MPTP formation and that it requires extracellular calcium entry (Choo, Saafir, Mkumba, Wagner & Jobe, 2012), suggesting that ROS generation can lead to PS exposure, but it is not fundamental. Here I have shown that hyperglycaemia significantly increases mitochondrial membrane depolarisation (Fig. 9D) and has no significant effect on ROS generation (Fig. 9B), further adding to the evidence that ROS generation is not essential for PS exposure.

It is well understood that extracellular calcium levels and elevated cytoplasmic calcium levels play an important role in the regulation of high-level PS exposure (Heemskerk, Vuist, Feijge, Reutelingsperger & Lindhout, 1997). However, Choo et al showed that mitochondrial membrane depolarisation is more closely linked to an increase in PS exposure than increased calcium signalling. And that MPTP formation driven PS exposure is not due to a change in cytosolic calcium levels (Choo, Saafir, Mkumba, Wagner & Jobe, 2012). This study has shown that hyperglycaemia inhibits platelet calcium signalling (Fig. 10C+D) which coincides with the idea that ROS generation is highly calcium dependent and MPTP formation and PS exposure are not. Taken together this data suggests that PS exposure can be initiated via a calcium dependent, ROS generation dependent pathway or a calcium independent, MPTP formation dependent pathway. This data contradicts the results shown by Liu et al, where they demonstrated that hyperglycaemia enhances calcium influx (Liu et al., 2008). This could potentially be explained by the fact that they incubated the platelets with 25 mM glucose for 60 minutes prior to stimulation whereas in the present study platelets were only incubated with glucose for 10 minutes and the fact that the cell-permeable diacylglycerol analog, 1-oleoyl-2-accetyl-sn-glycerol (OAG) was used to stimulate platelets as opposed to dual stimulation with thrombin and CRP-XL used in this study.

Aoki et al (Aoki et al., 1996) showed that patients with poorly glycaemic-controlled non-insulin-dependent diabetes had elevated platelet dependent thrombin generation. It would therefore be interesting to investigate the effect of hyperglycaemia on platelet-dependent thrombin generation. Agbani et al (Agbani, Williams, Hers & Poole, 2017) showed that an increase in the local thrombin concentration can lead to ballooning of the platelet membrane in platelets at the outer layer of the thrombus. If platelet derived thrombin generation is increased by hyperglycaemia then it would be interesting to research the effects of hyperglycaemia on platelet membrane ballooning.

Unlike several previous studies (table 2), I was unable to conclusively show that platelet incubation with high glucose can rescue the inhibitory effects of aspirin on

platelet aggregation (Gresele et al., 2010; Kobzar, Mardla & Samel, 2017; Le Guyader, Pacheco, Seaver, Davis-Gorman, Copeland & McDonagh, 2009; Russo et al., 2012). The data collected in this study shows a trend in an increase in platelet aspirin inhibited aggregation in the presence of 25 mM glucose but there is no statistical significance to this trend (Fig. 11A). For most donor's high glucose appeared to rescue the inhibitory effects of aspirin however for a minority of donors no effect of high glucose was seen. There are a few explanations as to why this might be; aggregation is a very variable assay in general which could explain large error bars and lack of significance. However, it is probably more likely to be due to varying blood glucose levels due to reasons previously mentioned. These different conditions could affect how sensitive the platelets are to the high glucose added during the experiment. A potential way around this would be to ask donors to avoid eating 12 hours prior to blood donation (don't have breakfast until after donation). A more practical solution may be to use a blood glucose monitor to test donor's glucose levels prior to donation and only take blood from donors with blood glucose levels within a pre-determined range.

TPO enhances platelet aggregation and PS exposure

This study has conclusively shown that TPO is able to enhance platelet function. In line with previous studies (Blair, Moore & Hers, 2015; Oda et al., 1996), washed human platelets treated with TPO showed elevated aggregation in response to agonist (Fig. 6A+B). The present study has shown, for the first time, that TPO is also able to significantly enhance platelet PS exposure (Fig. 7B). Interestingly, TPO only enhances PS when the platelets are stimulated for 5 minutes but has no effect when they are stimulated for 10 minutes - this suggests that TPO accelerates the rate at which platelets expose PS rather than increasing the maximum amount of PS that is exposed. Interestingly, when the platelets were treated with both a high concentration of glucose and TPO, no cumulative effect was seen (Fig. 8C). This would therefore suggest that both glucose and TPO enhance PS via a similar mechanism.

The present study has demonstrated that TPO causes an increase in mitochondrial membrane depolarisation (Fig. 9D). And figure 10 A+B shows that TPO has no effect platelet calcium mobilisation further adding to the theory that PS exposure does not rely on an increase in calcium signalling. Unlike hyperglycaemia, platelets treated with TPO expressed increased ROS generation (Fig. 9A) as well as mitochondrial membrane depolarisation and PS exposure.

Mitchell et al investigated the ability of c-Mpl receptor agonists (such as TPO) to protect platelets from apoptosis. They discovered that platelets did in fact show

resistance to apoptosis when treated with c-Mpl agonists, but this effect only appeared during the first week of therapy (Mitchell et al., 2014). This suggests that in vivo TPO is also able to accelerate platelet function or the effects of TPO are time limited. The authors concluded that c-Mpl agonists are able to induce transient signalling through the phosphoinositide 3-kinase (PI3-kinse) pathway leading to activation of the serine/threonine kinase PKB in vivo. PKB signals to over 100 proteins, but it's overall effect is pro-survival (Downward, 2004). Pasquet and colleagues have shown that the PI 3-kinase pathway is important in the priming action of TPO in response to collagen (Pasquet et al., 2000) and Blair et al have shown that platelets treated with TPO show significantly enhanced thrombus formation on a collagen-coated surface compared to platelets treated with vehicle (Blair et al., 2018). Interestingly, they demonstrated that p110α, a PI 3-kinase isoform, negatively regulates TPO-mediated enhancement of platelet function. However, as their experiments were carried out under non-coagulating conditions a link between the ability of TPO to enhance thrombin generation, demonstrated by Blair et al and its ability to increase platelet PS exposure cannot be made from these studies alone.

Combining all of this information and the data shown in the present study, I believe that this provides sufficient evidence to suggest that TPO is able to enhance the agonist-mediated pro-thrombotic exposure of PS by enhancing the generation of ROS leading to the formation of MPTPs and mitochondrial membrane depolarisation. Perhaps via the PI 3-kinase / PKB pathway. Interestingly, it has been reported that thromboxane production, calcium homeostasis and PI 3-kinase signalling are not involved in hyperglycaemia-related platelet hyperreactivity (Sudic, Razmara, Forslund, Ji, Hjemdahl & Li, 2006). This could perhaps explain the different effects of TPO and high glucose on intracellular calcium levels and ROS generation. The effects of TPO and glucose on platelet PS exposure demonstrated by this study are summarised in figure 16.

The mechanisms involved in platelet aggregation are distinct from those involved in platelet PS exposure. This study has clearly shown that TPO is able to enhance both aspects of platelet function whereas acute hyperglycaemia is only able to enhance the PS exposure aspect of platelet function.

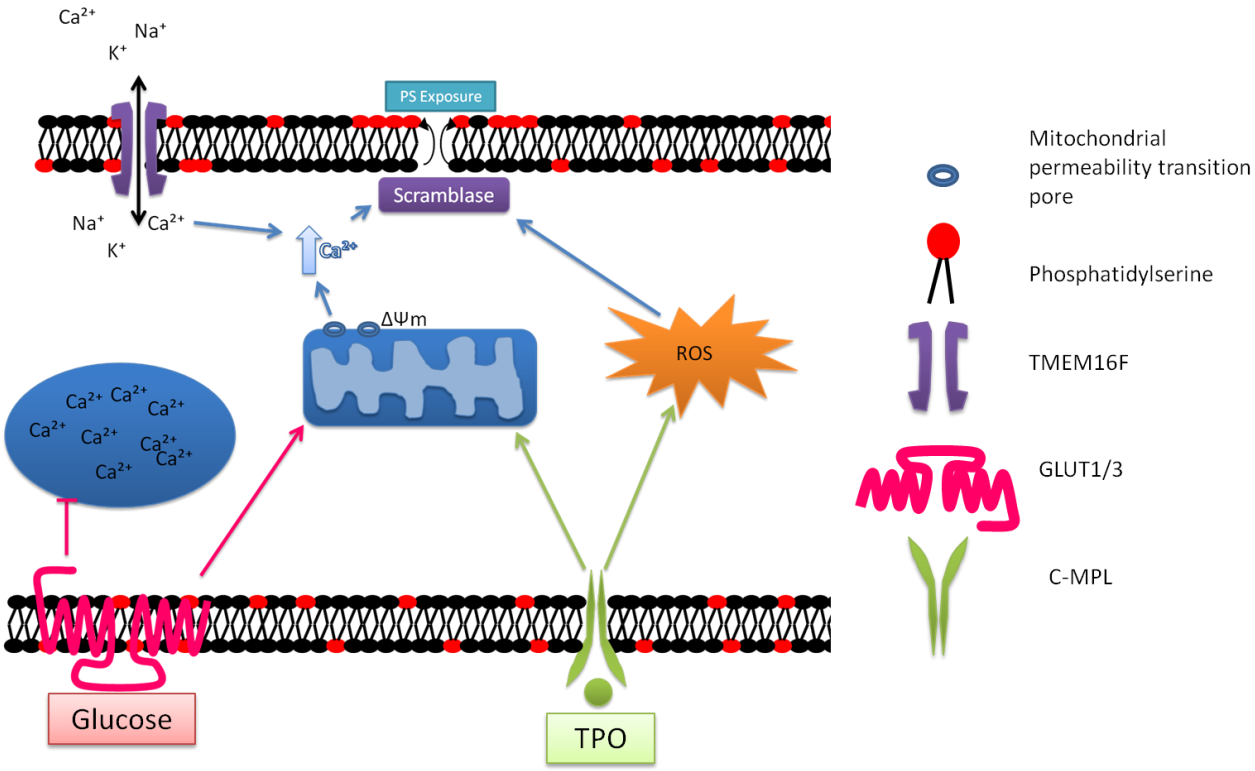

Figure 16: Summary figure showing the effect of Glucose and TPO on platelet PS exposure. Under highly stimulating conditions (e.g. Dual stimulation with CRP-XL and thrombin) platelets express greater levels of PS. The results of this study suggest that TPO is able to enhance this increase in PS exposure via an increase in ROS generation and an increase in mitochondrial permeability transition pore formation leading to an increase in calcium concentration and scramblase activity. Glucose on the other hand has no effect on platelet ROS generation and only causes an increase in platelet PS exposure via the depolarisation of the mitochondrial membrane resulting in an increase in calcium, leading to increased scramblase activity and PS exposure. Interestingly, this study has also shown that glucose reduces overall platelet calcium signalling, but calcium is known to be an important component of PS exposure and a direct result of mitochondrial membrane depolarisation. These contradictory results could be explained by the idea that glucose still causes an increase in local calcium signalling resulting in PS exposure, but an overall decrease in calcium signalling within the whole cell.

Metformin has no significant effect on platelet aggregation

Xin et al (Xin et al., 2016) have shown that metformin is capable of inhibiting platelet function. The results of this study shows a trend that suggests that metformin reduces platelet aggregation, however these results did not reach significance. It is likely that with a higher powered study it could be proved that metformin significantly inhibits platelet aggregation. However, unlike Xin et al, I found that metformin has no effect on integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression (Fig. 12E+F). Further investigation into the effects of metformin on platelet function is required.

Although the human proteome map shows the OCT1 transporter being present in human platelets (Fig. 4), there is still little evidence to suggest that metformin is able to cross the platelet cell membrane. Future work therefore needs to be carried out with regards to metformin and platelet function. I would suggest confirming that metformin is in fact able to enter platelets before continuing research into its effect on platelets. A potential way around this would be to look at the effects of phenformin, another biguanide, on platelet function as it has a much faster mechanism of action and is known to be able to cross the cell membrane (Bridges, Sirviö, Agip & Hirst, 2016). Phenformin was a drug produced before metformin but was quickly removed from the market due to toxicity reasons, mainly severe lactic acidosis (Assan, Heuclin, Girard, LeMaire & Attali, 1975). However, it would still be useful to investigate its effects on platelet function as a good indicator of metformin action. Firstly, in order to confirm phenformin is working I would suggest using western blotting to monitor AMPK regulation as phenformin has been shown to highly upregulate AMPK (Kim, Yang, Kim & Ha, 2016; Yang, Sha, Davisson & Qi, 2013).

Rosiglitazone inhibits platelet aggregation and enhances PS exposure.

In accordance with data shown by Liu et al (Liu, Park, Chang, Huh, Lee & Lee, 2016) this study has shown that platelet incubation with the anti-diabetic drug, rosiglitazone, significantly inhibits agonist-stimulated platelet aggregation (Fig. 13). Platelets treated with rosiglitazone showed reduced integrin activation and p-selectin expression, however this did not reach significance (Fig. 14A+B) this is probably due to the fact that this study was under-powered and further analyses need to be performed. Interestingly, the presence of rosiglitazone is enough to inhibit the enhancement of aggregation seen by TPO (Fig. 13C), suggesting that even in a hyper reactive state rosiglitazone is able to inhibit platelet aggregation.

On the contrary, rosiglitazone significantly enhanced platelet PS exposure (Fig. 15A+B) and increased platelet generation of ROS (Fig. 15C). Surprisingly, this study has also shown that treatment with rosiglitazone causes a significant reduction in mitochondrial membrane depolarisation (Fig. 14D). This is a strange phenomenon because both agonist-mediated and apoptotic PS exposure supposedly require mitochondrial membrane depolarisation. This would therefore suggest that PS exposure can be initiated by a calcium-dependent ROS generation pathway or by a calcium independent mitochondrial membrane depolarisation pathway. In order to confirm this it would be interesting to investigate the effects of rosiglitazone on platelet calcium mobilisation.

Contradictory to the data confirming the ability of rosiglitazone to inhibit platelet function, this study has also shown that rosiglitazone is able to significantly enhance platelet function in terms of ROS generation and PS exposure. This could provide a potential mechanism behind the increased risk of cardiac event seen in patients prescribed rosiglitazone (Cheng, Gao & Li, 2018).

Future works

A better understanding of the mechanism behind glucose entry into the platelet and information on time scale could be investigated using 2-NBDG, a fluorescent glucose analogue (Zou, Wang & Shen, 2005), which can be used to monitor glucose uptake into platelets using flow cytometry. GLUT3 translocation could also be monitored using a fluorescently tagged GLUT3 antibody.

Platelets in patients with diabetes are not simply exposed to high levels of blood glucose but there are many other factors that could be contributing to the increased platelet hyperreactivity observed. I would be very interested in investigating the effects of glucose and TPO on diabetic mice and/or patients and compare the results to healthy controls.

In order to further the work on the ability of TPO to enhance platelet function I would suggest investigating the effect of TPO on thrombin generation and membrane ballooning. As an increase in PS exposure should lead to an increase in thrombin generation which in turn would lead to an increase in membrane ballooning. This would also be worth investigating in terms of the effect of hyperglycaemia on platelet function. One way of testing the theory that the increase in PS exposure is PI 3-kinase dependent would be to repeat the same experiments but in the presence of the PI 3-kinase inhibitor wortmannin. Using western blotting to monitor PI 3-kinase activity, PKB and other potential downstream signalling events in order to reveal the mechanism of action. It would also be interesting to investigate whether PS exposure can be mediated by ROS generation or mitochondrial membrane depolarisation independently of each other. This could be done by stimulating PS exposure whilst, for example, simultaneously inhibiting platelet ROS generation. In addition to these suggested experiments, animals' models could be used to investigate the effects of hyperglycaemia and increased serum levels of TPO in vivo.

Conclusion

Increased serum levels of TPO and glucose are associated with various clinical conditions and will contribute to platelet hyperreactivity and consequently increased

risk of cardiovascular disease. Further research into the mechanism behind this is required in order to address anti platelet resistance seen in diseases such as diabetes. Metformin has shown some promising evidence to suggest its capabilities as an anti-platelet therapy alternative, however confirmation of its ability to enter platelets and inhibit function is necessary. Rosiglitazone is still used as an anti-diabetic treatment in the US but has been removed from the European market due to an increased risk of cardiovascular disease, the results from this study may suggest a potential explanation for this observed life-threatening side effect.

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